Universidade de Aveiro Departamento de Ciências Médicas
2015

Ana Raquel Gonçalves Pandeirada

Associação entre polimorfismos genéticos comuns do metabolismo lípidico e o risco de adenocarcinoma colo-retal esporádico

Association between common lipid metabolism genes polymorphisms and sporadic colorectal adenocarcinoma risk

Universidade de Aveiro Departamento de Ciências Médicas
2015

Ana Raquel Gonçalves Pandeirada

Associação entre polimorfismos genéticos comuns do metabolismo lípidico e o risco de adenocarcinoma colo-retal esporádico

Association between common lipid metabolism genes polymorphisms and sporadic colorectal adenocarcinoma risk

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina molecular, realizada sob a orientação científica da Professora Mestre Sandra dos Santos Caramujo Balseiro, Professora assistente na Escola Superior de Saúde Dr. Lopes Dias do Instituto Politécnico de Castelo Branco, e sob a co-orientação da Professora Doutora Odete Abreu Beirão da Cruz e Silva, Professora auxiliar no Departamento de Ciências Médicas da Universidade de Aveiro.

Este trabalho contou com a colaboração do Instituto de Anatomia Patológica e Patologia Molecular da Faculdade de Medicina da Universidade de Coimbra e foi financiado pelos projetos da CIMAGO (projeto nº17/2009) e do GAI (projeto LCarvalho.GAI2015) da FMUC.

Dedico esta dissertação à memória da minha mãe e da minha madrasta.

o júri

Presidente

Professora Doutora Ana Gabriela da Silva Cavaleiro Henriques
Prof. Associada do Departamento de Ciências Médicas,
Universidade de Aveiro

Professora Mestre Sandra dos Santos Caramujo Balseiro
Prof. Assistente da Escola Superior de Saúde Dr. Lopes Dias,
Instituto Politécnico de Castelo Branco

Professora Doutora Lina Maria de Rodrigues Carvalho
Prof. Agregada da Faculdade de Medicina,
Universidade de Coimbra

Agradecimentos

A realização desta dissertação de mestrado foi possível graças ao apoio de várias pessoas imprescindíveis, as quais gostaria de deixar o meu agradecimento.

Quero começar por agradecer à minha orientadora, Sandra Balseiro por todo o apoio, colaboração e partilha de ensinamentos durante a realização desta tese. Pelas opiniões e críticas, e também pela sua amizade e enorme paciência.

À Maria João, Natália, Patrícia, Sofia, Tiffany e Zé pedro por todo o apoio no laboratório mas também pela sua amizade. Também quero agradecer à Maria pela bondade que demonstrou sempre que tinha alguma dúvida e por me incentivar nas decisões mais difíceis.

À Diretora do Instituto de Anatomia Patológica da Faculdade de Medicina da Universidade de Coimbra pela oportunidade de realizar a minha tese de mestrado neste serviço.

Ao meu Pai (e mãe) um muito obrigado por todo o apoio incondicional e incentivo na superação dos obstáculos que surgiram durante este percurso. Principalmente por toda a sua positividade e força que me transmite todos os dias. Também por todas as "ferramentas" e ensinamentos transmitidos permitindo ser a pessoa que sou hoje.

À minha mãe e à minha madrasta, que apesar de estarem presentes apenas em pensamento sei que me dão força todos os dias para ultrapassar os momentos mais difíceis e lutar pelos meus sonhos. Em especial à minha madrasta, Lucinda, que sempre acreditou em mim e ensinou-me a ver sempre algo bom mesmo nas piores coisas e fases da vida, mostrando-me que para se conseguir algo tem que se lutar.

Aos meus avós, por todo o aconchego.

Ao meu namorado, David, por todo o carinho, companheirismo e apoio durante este percurso, essencialmente, por toda a compreensão que teve comigo nestes últimos meses, pois o tempo era sempre contado. Também por me apoiar em todas as decisões, mesmo nas mais difíceis.

Às minhas grandes amigas, o meu grande pilar, Lurdes e Rita, pela sua enorme amizade, ajuda, concelhos, disponibilidade, gargalhadas nos dias mais difíceis e por provarem todos os dias que as amizades verdadeiras e puras realmente existem. Quero agradecer por apesar da distância a que vivemos umas das outras, estão sempre presentes e prontas para ouvir os meus desabafos.

À Cristiana, Bela, Mariana, Catarina, Marcela, Inês e Patrícia por toda a amizade e apoio.

À CIMAGO (projeto nº17/2009) e ao GAI (projeto LCarvalho.GAI2015) pelo financiamento.

palavras-chave

Adenocarcinoma colo-rectal esporádico, metabolismo lípidico, APOA1, APOB, APOC3, APOE, CETP, NPY, PON1, PPARG, polimorfismos comuns.

resumo

Os lípidos podem modular o risco de desenvolver Adenocarcinoma Colo-rectal Esporádico (ACE), uma vez que alterações nas vias do metabolismo e do transporte lipídico podem influenciar diretamente a absorção do colesterol e dos lípidos pelas células do cólon e indiretamente a síntese de espécies reativas de oxigénio (ERO) no reto, devido à acumulação de lípidos. O metabolismo lipídico é regulado por várias proteínas (APOA1, APOB, APOC3, APOE, CETP, NPY, PON1, PPARG) que podem influenciar o metabolismo e o transporte de lípidos. Tem sido reportados nestes genes, vários polimorfismos comuns (SNP) que podem alterar a sua função e/ ou a expressão, causando um desequilíbrio no metabolismo dos lípidos. Estas alterações genéticas podem influenciar o desenvolvimento de ACE, no entanto a maioria dos polimorfismos nunca foram estudados nesta patologia. Além disso, existem resultados contraditórios entre alguns dos polimorfismos e o risco de ACE. Deste modo, o objetivo deste estudo foi explorar e descrever o efeito dos polimorfismos comuns de genes associados ao metabolismo lipídico (*APOA1* -75 G>A; *APOB* R3500Q; *APOC3* C3175G, *APOC3* T3206G; *APOE* Cys112/158Arg; *CETP* G279A, *CETP* R451Q; *NPY* Leu7Pro; *PON1* Q192R; *PPARG* Pro12Ala) no ACE e a sua relação com o risco de desenvolver ACE.

A genotipagem dos polimorfismos comuns de genes associados ao metabolismo lipídico (*APOA1* -75 G>A; *APOB* R3500Q; *APOC3* C3175G, *APOC3* T3206G*; APOE* Cys112/158Arg*; CETP* G279A*, CETP* R451Q; *NPY* Leu7Pro; *PON1* Q192R; *PPARG* Pro12Ala*)* foi efetuada pela técnica de PCR-SSP, a partir de biópsias incluídas em parafina e fixadas em formol de 100 indivíduos saudáveis e de 68 indivíduos com ACE.

Os genótipos mutantes *APOA1* -75AA (32% vs 12%; p=0.001; OR=3.51; 95% CI 1.59-7.72); *APOB* 3500AA (7% vs 0%; p=0.01); *APOC3* 3175GG (19% vs 2%; p=0.0002; OR=11.58; 95% CI 2.52-53.22), *APOC3* 3206GG (19% vs 0%; p<0.0001); *CETP* 279AA (12% vs 1%; p=0.003; OR=13.20; 95% CI 1.61-108.17), *CETP* 451AA (16% vs 0%; p<0.0001); *NPY* 7CC (15% vs 0%; p<0.0001); *PPARG* 12GG (10% vs 0%; p=0.001); e o genótipo heterozigótico *PON1* 192AG (56% vs 22%; p<0.0001; OR=4.49; 95% CI 2.29-8.80) demonstraram estar associados à prevalência de ACE. Enquanto, o haplótipo mutante *APOE* E4/E4 (0% vs 8%; p=0.02) mostrou ter um efeito protetor no ACE. Adicionalmente, também foram encontradas diferenças entre a prevalência e a localização tumoral (cólon ou reto) para os genótipos *APOB* 3500GA*, APOC3* 3206TG*, CETP* 279AA e para o alelo *PPARG* 12Ala.

Estes resultados sugerem uma associação positiva entre a maioria dos polimorfismos genéticos comuns estudados envolvidos no metabolismo lipídico e a prevalência de ACE. A desregulação dos genes *APOA1, APOB, APOC3, CETP, NPY, PON1, PPARG* poderá estar associada com a diminuição dos níveis plasmáticos de colesterol e o aumento de ERO na mucosa do colon e do reto. Para além disso, estes resultados também suportam a hipótese de que o CCR esta relacionado com a diminuição da absorção intestinal e com o aumento da produção de ácidos biliares secundários. Adicionalmente, os polimorfismos estudados podem desempenhar um importante papel como biomarcadores de suscetibilidade para ACE.

keywords

Sporadic colorectal adenocarcinoma, lipid metabolism, APOA1, APOB, APOC3, APOE, CETP, NPY, PON1, PPARG, common polymorphisms

abstract

Lipids can modulate the risk of developing sporadic colorectal adenocarcinoma (SCA), since alterations into lipid metabolism and transport pathways influence directly cholesterol and lipids absorption by colonic cells and indirectly reactive oxygen species (ROS) synthesis in rectum cells due to lipid accumulation. Lipid metabolism is regulated by several proteins APOA1, APOB, APOC3, APOE, CETP, NPY, PON1 and PPARG that could influence both metabolism and transport processes. Is been reported that several common single-nucleotide polymorphisms (SNPs) in these genes could influence their function and/or expression, changing lipid metabolism balance. Thus, genetic changes in those genes can influence SCA development, once the majority of them were never studied in this disease. Furthermore, there are contradictory results between some studied polymorphisms and SCA risk. Thus, the aim of this study was to explore and describe lipid metabolism-associated genes common polymorphisms (*APOA1* -75 G>A; *APOB* R3500Q; *APOC3* C3175G, *APOC3* T3206G; *APOE* Cys112/158Arg; *CETP* G279A, *CETP* R451Q; *NPY* Leu7Pro; *PON1* Q192R; *PPARG* Pro12Ala) status among SCA, and their relationship with SCA risk.

Genotyping of common lipid metabolism genes polymorphisms (*APOA1* -75 G>A; *APOB* R3500Q; *APOC3* C3175G, *APOC3* T3206G; *APOE* Cys112/158Arg; *CETP* G279A, *CETP* R451Q; *NPY* Leu7Pro; *PON1* Q192R; *PPARG* Pro12Ala) were done by PCR-SSP techniques, from formalin-fixed and paraffin-embedded biopsies of 100 healthy individuals and 68 SCA subjects.

Mutant genotypes of *APOA1* -75AA (32% vs 12%; p=0.001; OR=3.51; 95% CI 1.59-7.72); *APOB* 3500AA (7% vs 0%; p=0.01); *APOC3* 3175GG (19% vs 2%; p=0.0002; OR=11.58; 95% CI 2.52-53.22), *APOC3* 3206GG (19% vs 0%; p<0.0001); *CETP* 279AA (12% vs 1%; p=0.003; OR=13.20; 95% CI 1.61-108.17), *CETP* 451AA (16% vs 0%; p<0.0001); *NPY* 7CC (15% vs 0%; p<0.0001); *PPARG* 12GG (10% vs 0%; p=0.001); and heterozygote genotype *PON1* 192AG (56% vs 22%; p<0.0001; OR=4.49; 95% CI 2.29-8.80) were found associated with SCA prevalence. While, *APOE* E4/E4 (0% vs 8%; p=0.02) mutant haplotype seemed to have a protective effect on SCA. Moreover, it also been founded differences between *APOB* 3500GA, *APOC3* 3206TG, *CETP* 279AA genotypes and *PPARG* 12Ala allele prevalence and tissue localization (colon vs rectum).

These findings suggest a positive association between most of common lipid metabolism genes polymorphisms studied and SCA prevalence. Dysregulation of *APOA1*, *APOB*, *APOC3*, *CETP*, *NPY*, *PON1* and *PPARG* genes could be associated with lower cholesterol plasma levels and increase ROS among colon and rectum mucosa. Furthermore, these results also support the hypothesis that CRC is related with intestinal lipid absorption decrease and secondary bile acids production increase. Moreover, the polymorphisms studied may play an important role as biomarkers to SCA susceptibility.

Index

Abbreviations..... 16

Capther I: Introduction 20

1. Introduction 22

1.1. Epidemiology of colorectal cancer 23

1.1.1. Incidence and mortality 23

1.1.2. Etiology 25

1.2. Histological classification 26

1.3. Environmental and genetic risk factors 28

1.4. Lipid metabolism genes and CRC 29

1.4.1. Apolipoprotein A (APOA1) 32

1.4.2. Apolipoprotein B (APOB) 33

1.4.3. Apolipoprotein C3 (APOC3) 34

1.4.4. Apolipoprotein E (APOE) 36

1.4.5. Cholesterol ester transfer protein (CETP) 37

1.4.6. Neuropeptide Y (NPY) 38

1.4.7. Paraoxonase 1 (PON1) 39

1.4.8. Peroxisome proliferator-activated receptor gamma (PPARG) 40

1.5. Lipid transport and metabolism genes in Sporadic Colorectal Cancer (SCA) 41

Capther II: Materials and methods 44

2. Materials and methods 46

2.1. Biological samples 46

2.2. DNA extraction 47

2.2.1. Analysis of concentration and quality of the extracted DNA 48

2.3. Genotyping 48

2.3.1. Amplification by *Polymerase Chain Reaction-Sequence Specific Primers* (PCR-SSP) 49

2.3.2. Electrophoresis in agarose gel 50

2.4. Statistical analysis 51

2.4.1. Analysis of purity and concentration of DNA 51
2.4.2. Allele and genotype frequencies of polymorphisms 51
Capther III: Results 42
3. Results 54
3.1. Clinical pathology data 54
3.2. Analysis of DNA purity and concentration 54
3.3.1. *APOA1* -75 G>A*, APOB* R3500Q*, APOC3* C3175G, *APOC3* T3206G and *APOE* Cys112/158Arg distribution among SCA and controls 56
3.3.2. *CETP* G279A and *CETP* R451Q (G>A) polymorphisms distribution among SCA and controls 59
3.3.3. *NPY* Leu7Pro, *PON1* Q192R (A>G) and *PPARG* Pro12Ala polymorphisms distribution among SCA and controls 62
3.4. Lipid metabolism genes polymorphisms in SCA stratified by clinical pathological features 65
3.4.1. Gender 65
3.4.2. Tumour localization 68
Capther IV: Discussion 74
4. Discussion 76
4.1. Influence of common lipid metabolism genes polymorphism among SCA and controls 78
4.1.1. Influence of APOA1 polymorphism in SCA 79
4.1.2. Influence of APOB polymorphism in SCA 80
4.1.3. Influence of APOC3 polymorphisms in SCA 81
4.1.4. Influence of APOE polymorphisms in SCA 81
4.1.5. Influence of CETP polymorphisms in SCA 82
4.1.6. Influence of NPY polymorphism in SCA 83
4.1.7. Influence of PON1 polymorphism in SCA 83
4.1.8. Influence of PPARG polymorphism in SCA 84
4.2. Influence of lipid transport and metabolism genes polymorphisms in SCA 84
4.3. Gender 85

4.4. Distinction between colonic and rectal adenocarcinomas..... 85
4.5. Future perspectives 86
4.6. Study limitations 87
Capther V: Conclusion 88
5. Conclusion 90
Capther VI: References 92
6. References 94
Capther VII: Appendix 112
7. Appendix 114

Figures Index

Figure 1. Epidemiology. **(A)** Colorectal cancer incidence and mortality to both genres in the worldwide. **(B)** News cases and deaths of different cancers in Portugal. 24

Figure 2. Schematic classification of colorectal cancers. 26

Figure 3. Schematic of high density lipoproteins (HDL) and low density lipoproteins (LDL) structure. Apo indicates apolipoprotein. 30

Figure 4. Schematic representation of exogenous cholesterol transport pathway. 31

Figure 5. Schematic representation of endogenous cholesterol transport pathway. 31

Figure 6. Schematic representation of reverse cholesterol transport pathway. 32

Figure 7. Cytogenetic localization of *APOA1* gene. 33

Figure 8. Cytogenetic localization of *APOB* gene. 34

Figure 9. Representation APOC3 effects in lipoprotein metabolism pathways. APOC3 can modulate enzymes that are involved in cholesterol transport from extrahepatic tissues to the liver. APOC3 inhibits (↓) LCAT activity, VLDL TGs clearance and TRLs lipolysis. Furthermore, APOC3 also stimulates (↑) CETP activity. 35

Figure 10. Cytogenetic localization of *APOC3* gene. 36

Figure 11. Cytogenetic localization of *APOE* gene. 37

Figure 12. Cytogenetic localization of *CETP* gene. 38

Figure 13. Cytogenetic localization of *NPY* gene. 39

Figure 14. Effect of dietary components on regulation of PON1 activity and its interaction with genetic factors. 39

Figure 15. Cytogenetic localization of *PON1* gene. 40

Figure 16. Cytogenetic localization of *PPARG* gene. 41

Figure 17. (A), **(B)** Histologic image of normal colonic mucosa: 40x HE (Haematoxylin-eosin), 200x HE; **(C)**, **(D)** Histological image of well differentiated sporadic colorectal adenocarcinoma (According to WHO criteria). 100x HE; 200x HE. Images from IAP-FMUC. 46

Figure 18. Allele and genotype frequencies of apolipoproteins genes polymorphisms among the SCA and controls subjects: **(A)** Allele frequencies and **(B)** Genotype frequencies of *APOA1* -75 G>A; **(C)** Allele frequencies and **(D)** Genotype frequencies of *APOB* R3500Q;

(E) Allele frequencies and **(F)** Genotype frequencies of *APOC3* C3175G; Genotype frequencies of **(G)** *APOC3* T3206G and **(H)** *APOE* Cys112/158Arg (* p≤0.05; **p≤0.001; ***p≤0.0001). 57

Figure 19. Allele and genotype frequencies of *CETP* gene polymorphisms among SCA and controls subjects: **(A)** Allele frequencies and **(B)** Genotype frequencies of *CETP* G279A; **(C)** Allele frequencies and **(D)** Genotype frequencies of *CETP* R451Q (G>A) (**p≤0.001; ***p≤0.0001). 60

Figure 20. Allele and genotype frequencies distribution among SCA and controls subjects: **(A)** Allele and **(B)** Genotype frequencies of *NPY* Leu7Pro polymorphism; **(C)** Allele and **(D)** Genotype frequencies of *PON1* Q192R (A>G) polymorphism; **(E)** Allele and **(F)** Genotype frequencies of *PPARG* Pro12Ala polymorphism (*p≤0.05; **p≤0.001; ***p≤0.0001). 63

Figure 21. Allele frequencies of *PPARG* Pro12ala in **(A)** Colon SCA and Rectum SCA **(B)** Rectum SCA and control subjects (*p≤0.05; **p≤0.001). 70

Figure 22. Genotype frequencies in Colon SCA and Rectum SCA to **(A)** *APOB* R3500Q, **(B)** *APOC3* T3206G and **(C)** *CETP* G279A (*p≤0.05). 71

Figure 23. Diagram of SCA progression and development from increase of bile acid levels caused by genetic factors (SNP's) and high fat diet. 77

Tables Index

Table 1. Estimated incidence, mortality and 5-year prevalence for colorectal cancer, in World, Europe and Portugal to men and women. 24

Table 2. Histological classification according to the 7th version (2012) from World Health Organization (WHO). 27

Table 3. Clinical pathological data SCA and controls. 47

Table 4. Lipid metabolism common polymorphisms analyzed in this study. 49

Table 5. Protocol of the program to amplification by PCR-SSP (*Polymerase Chain Reaction – Sequence Specific Primers*), from Genebox, Cantanhede – Portugal. 50

Table 6. Gender distribution among colon and rectum groups..... 54

Table 7. Means and standard-deviations of DNA samples purity and concentration values 55

Table 8. Allele frequencies distribution of *APOA1* -75 G>A*, APOB* R3500Q*, APOC3* C3175G*, APOC3* T3206G and *APOE* Cys112/158Arg among SCA and control subjects. 58

Table 9. Genotype frequencies distribution of *APOA1* -75 G>A*, APOB* R3500Q*, APOC3* C3175G*, APOC3* T3206G and *APOE* Cys112/158Arg among SCA and control subjects. 59

Table 10. Allele frequencies distribution of *CETP* G279A and *CETP* R451Q (G>A) among SCA and control subjects. 61

Table 11. Genotype frequencies distribution of *CETP* G279A and *CETP* R451Q (G>A) among SCA and control subjects. 61

Table 12. Allele frequencies distribution of *NPY* Leu7Pro, *PON1* Q192R (A>G) and *PPARG* Pro12Ala among SCA and control subjects. 64

Table 13. Genotype frequencies distribution of *NPY* Leu7Pro, *PON1* Q192R (A>G) and *PPARG* Pro12Ala among SCA and control subjects. 64

Table 14. Allele frequencies distribution between genders. 65

Table 15. Genotype frequencies distribution between genders. 66

Table 16. Allele frequencies distribution in colon versus rectum SCA. 68

Table 17. Allele frequencies of *PPARG* Pro12Ala distribution among colon SCA and controls subjects. 70

Table 18. Allele frequencies of *PPARG* Pro12Ala distribution among rectum SCA and controls subjects. 70
Table 19. Genotype frequencies distribution in colon and rectum SCA samples. 72
Table 20. Hardy-Weinberg Equilibrium to common lipid metabolism genes polymorphisms in control population. 114

Abbreviations

ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
APC	*Adenomatous polyposis coli*
APOA1	Apolipoprotein 1
APOB	Apolipoprotein B
APOC3	Apolipoprotein C3
APOE	Apolipoprotein E
BAs	Bile acids
BAs I	Bile acids primary
BAs II	Bile acids secundary
BMI	Body mass index
CETP	Cholesterol esters transport protein
CI	Confidence interval
CIN	Chromosomal instability
CM	Chylomicrons
CRC	Colorectal cancer
CVD	Cardiovascular diseases
EGFR	Epidermal growth factor receptors
FAP	Familiar adenomatous polyposis
FDB	Familial defective apolipoprotein B-100
FFA	Free fatty acid
FFPE	Formalin-fixed and paraffin-embedded
FH	Familial hypercholesterolemia
GWAS	Genome-wide association studies
HE	Haematoxylin-eosin
HDL	High density lipoprotein
HDL-C	High density lipoprotein - cholesterol
IDL	Intermediary density lipoprotein

LDL	Low density lipoprotein
LDL-C	Low density lipoprotein-cholesterol
LDL-R	Low density lipoprotein receptor
LOH	Loss of heterozygosity
LOOHs	Hydroperoxides
LPL	Lipoprotein lipase
MMR	Mismatch repair
MSI	Microsatellite instability
NF-B	Nuclear factor kappa B
NPY	Neuropeptide y
OD	Optical density
OR	Odds ratio
PCR-SSP	Polymerase chain reaction – sequence specific primers
PKC	Protein kinase C
PON1	Paraoxonase 1
PPARG	Peroxisome proliferator-activated receptor gamma
PTEN	Phosphatase and tensin homolog
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Relative risk
SCA	Sporadic colorectal adenocarcinoma
SNP	Single-nucleotide polymorphism
SR-B1	Scavenger receptor class B1
STAT3	Signal transducer and activator of transcription 3
VLDL	Very low density lipoprotein
VLDL-C	Very low density lipoprotein-cholesterol
WHO	World health organization
Wt	Wild-type
TAE	Tris-Acetate-EDTA
TG	Triglyceride

TRLs	Triglyceride rich lipoproteins
UV	Ultra-violet

Chapter I
Introduction

1. Introduction

Colorectal cancer (CRC) is the fourth most common cancer cause of death worldwide.[1] CRC is a serious health problem, a challenge for research, and a model for studying the molecular mechanisms involved in its development.[2] This pathology comprehends to types of tissue structures colon (constituted by four sections, such as, ascending colon located on the right side, transverse colon, descending colon located on the left side and sigmoid colon) and rectum.[3] Colon mucosa has multiple tubular invaginations called "crypts of Lieberkuhn", that are wide, deep, and numerous, along the surface of its epithelium and in which the regeneration of the epithelium takes place. Because of its biological nature, the colon has a high level of cellular regeneration and a physical, chemical, and biological nature, which increases the possibility of developing several pathologies, including cancer.[2] Rectum is located between sigmoid colon and anal canal, it has among 12 to 16 cm long and can be subdivided in three parts: upper rectum, middle rectum and lower rectum. Histologically is identical with colon, constituted by mucosa, submucosa, muscularis and serosa. Mucosa is constituted by typical intestinal epithelium. At anal transitional zone the columnar epithelium flattens becomes a stratified squamous non-keratinized epithelium. Submucosa contains loose connective tissue with blood vessels, lymph follicles and the Meissner's plexus. Lastly, muscularis has the typical inner circular and outer longitudinal musculature between which the Auerbach's plexus.[4]

CRC development involves one or more genetically altered cells and requires many years to progress[5], with a multistep process that affords nutrients an opportunity to modify the evolution of the disease. Many different dietary nutrients, such as lipids, contact epithelial cells in the colonic crypts both from the lumenal contents of the colon and from the basolateral epithelial cell membranes, thereby influencing developmental processes in both normal and transformed colonic epithelial cells.[6] On other hand, rectum tissues are in contact with diet waste, including lipids that are not transported into blood stream. There are vary genetic and environmental factors involved in CRC development, namely, genes involved in metabolic processes, including lipid metabolism.[7,8] There are many authors that referred an association between CRC, low cholesterol intestinal

absorption[9,10,11] and high secondary bile acids excretion thru feces[12]. Additionally, single-nucleotide polymorphisms (SNP'S) in lipid metabolism genes also are determinants potentials involved in progression of CRC, being this topic more development in environmental and genetic factors.[8]

1.1. Epidemiology of colorectal cancer

1.1.1. Incidence and mortality

Every year more than 1,2 million patients are diagnosed with CRC , and more 600 000 die in the world with CRC.[1] CRC represents 12.9% of all newly diagnosed cancers and is responsible for 12.2% of all cancer deaths, in Europe.[13] CRC is the third most common cancer in men (746,000 cases, 10.0% of the total) and the second in woman (614,000 cases, 9.2% of the total) worldwide. However, CRC is not uniformly common throughout the world, about 55% of the cases occur in more developed countries (Figure 1.A). But, its mortality is higher in less developed regions because medical resources privation (Figure 1.A).[14]

In Portugal, CRC incidence also has been increased in recent decades; it presents a large variety, depending on the geographical region. According to 2012 data, the CRC is the cancer with higher incidence and mortality among both genders (Figure 1.B).[14] Furthermore, deaths by CRC increased 3% per year between 2000 and 2005 and the overall survival rates at 1, 3 and 5 years are estimated at 73%, 55% and 46%, respectively.[13]

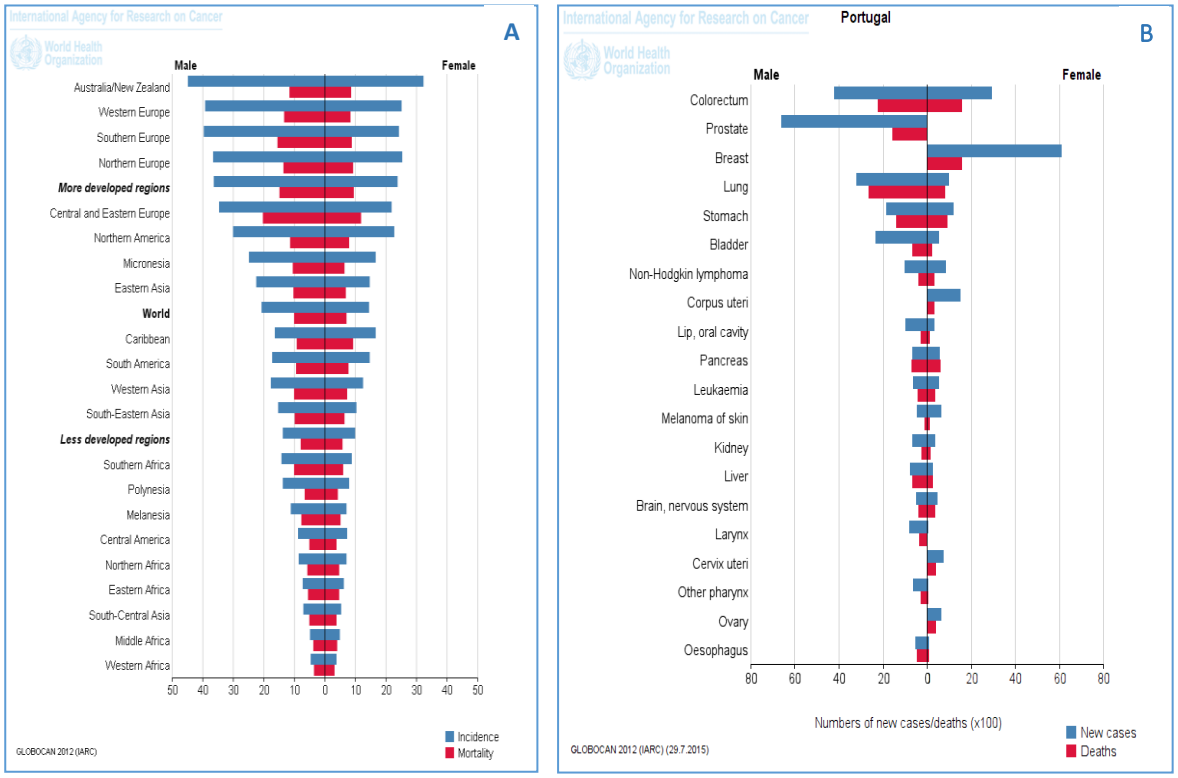

Figure 1. Epidemiology. **(A)** Colorectal cancer incidence and mortality to both genres in the worldwide. **(B)** News cases and deaths of different cancers in Portugal.[14]

Generally, according to the Globocan 2012 data, in Portugal, Europe and worldwide there is a high incidence and mortality for CRC, it can be stated that approximately half of those who diagnose this tumour die. Also, is estimated that the prevalence of CRC in both genders should duplicate in a five years range (Table 1).[15]

Table 1. Estimated incidence, mortality and 5-year prevalence for colorectal cancer, in World, Europe and Portugal to men and women.[15]

	Region	Gender	Number of cases		
			Incidence	Mortality	5-year prevalence
Colorectal cancer	World	Men	746298	373639	1953431
		Women	614304	320294	1590151
	Europe	Men	241813	113246	656384
		Women	205323	101620	547559
	Portugal	Men	4209	2240	11581
		Women	2920	1557	8032

The rapid increases in the previously low-risk countries, such as Spain and several countries in eastern Europe and east Asia, have been noted, which have been ascribed to changes in dietary patterns and risk factors towards a so-called western lifestyle.[16,17] However, in the USA and several other high income countries, incidence has stabilized or started to decrease, probably because of increased use of sigmoidoscopy and colonoscopy with polypectomy.[3,18,19,20] In several high-income countries and countries of east Asia and eastern Europe, mortality has been decreasing since the 1980s, probably because of improved early detection and treatment.[18] Nevertheless, rates are still increasing in countries or areas with poor health-care resources, including countries in Central and South America[21] and rural areas in China[22].

The increase of CRC cases number in the last thirty years is strongly linked to changes in lifestyle and exposure to carcinogens. The gradual abandonment of the Mediterranean diet[23], increased caloric intake, increased intake of animal fats[24] and red meats[25], increased obesity and sedentary lifestyle[26], as well as tobacco consumption are strong contributors to the increase in CRC incidence.[19]

1.1.2. Etiology

There are three different types of CRC development that can be distinguished: sporadic; family; and hereditary form. About 60-85% of CRC cases are sporadic type that occurs in patients with advanced ages. Sporadic cancers are caused by genetic abnormalities in tumour suppressor genes and oncogenes that give cells an evolutionary advantage over their neighbors. However, hereditary and familial CRC forms, are responsible for 5-10% and 10-30% of the cases, respectively.[2] Hereditary non-polyposis colorectal cancer (Lynch Syndrome) is the most common form of hereditary CRC. It is inherited in autosomal dominant fashion, its clinical consequences develop from germline mutations in mismatch repair (MMR) genes, where occurs the inactivation of the repair system.[27] Other hereditary syndrome, is familiar adenomatous polyposis (FAP) that is characterized by numerous of adenomatous colorectal polyps. FAP is an autosomal dominant hereditary cancer syndrome caused by a germline mutation in the *APC* gene

(*adenomatous polyposis coli*), being the first molecular event in the progress of sporadic colorectal adenocarcinoma.[28,29,30]

These CRC forms can develop through different genetic pathways. The chromosomal instability (CIN) pathway occurs in 80-85% of the CRC (Figure 2), known as the adenoma-carcinoma sequence.[27,31,32,33] In inherited and sporadic tumours it has been demonstrated several alterations in specific genes, involved acquisition of a new or increased oncogenes function and tumour suppressor genes function loss. The genetic changes include tumour suppressor genes *APC* (chromosome region 5q21) and *TP53* (chromosome region 17p13) function loss, proto-oncogenes (*K-ras*) activation and heterozygosity for the long arm of chromosome 18q (*DCC, SMAD2, SMAD4*) LOH loss.[5,34,35,36] Beyond this, aneuploidy, amplifications, and translocations are also common in these tumours. Other genetic pathway, present in 15% of CRC cases, is microsatellite instability (MSI) characterized by DNA mismatch repair (MMR) system proficiency loss (Figure 2).[27]

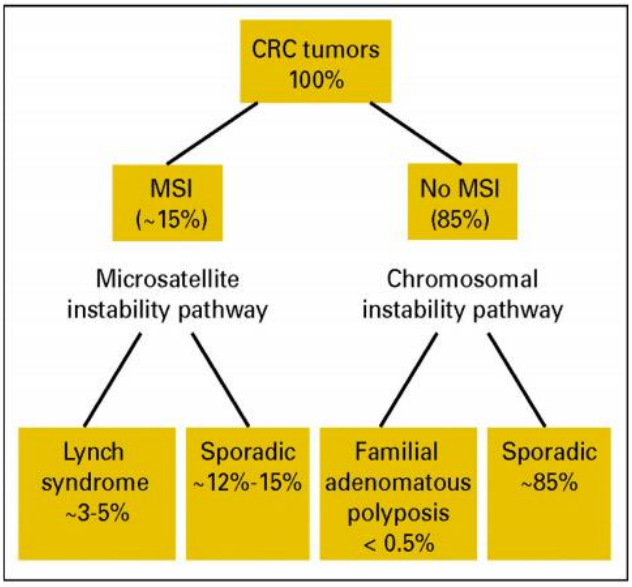

Figure 2. Schematic classification of colorectal cancers.[27]

1.2. Histological classification

More than 90% of CRC are adenocarcinoma's; its origin comes from colorectal mucosa epithelial cells. Conventional adenocarcinoma is characterized by glandular formation, which is the basis for histologic tumour grading. In well differentiated

adenocarcinoma's (Stage I) >95% of tumours are gland forming. Moderately differentiated adenocarcinoma's (Stage II) show 50-95% gland formation. Poorly differentiated adenocarcinoma's (Stage III) are mostly solid with <50% gland formation. In general, most colorectal adenocarcinoma's (±70%) are diagnosed as moderately differentiated. Well and poorly differentiated adenocarcinoma's account for 10% and 20%, respectively. Invasive colorectal adenocarcinoma's may be seen dysplasia in adjacent mucosa. Cells with high-grade dysplasia exhibit rounded nuclei, coarse chromatin, prominent nucleoli, and loss of nuclear polarity with nuclei no longer being oriented perpendicular to the basement membrane.[37,38] Histological classification of CRC is based upon the most recent classification version (7th edition, 2012) according to World Health Organization (WHO) (Table 2).[39]

Table 2. Histological classification according to the 7th version (2012) from World Health Organization (WHO).[39]

Histological classification according to WHO			
Epithelial tumours		**Non-epithelial tumours**	
Adenoma		Lipoma	
	Tubular	Leiomyoma	
	Villous	Gastrointestinal stromal tumour	
	Tubulovillous	Leiomyosarcoma	
	Serrated	Angiosarcoma	
		Kaposi sarcoma	
Intraepithelial neoplasia (dysplasia) associated with chronic inflammatory diseases		Malignant melanoma	
		Others	
	Low-grade glandular intraepithelial neoplasia		
	High-grade glandular intraepithelial neoplasia		
		Malignant lymphomas	
Carcinoma			Marginal zone B-cell lymphoma of MALT Type
	Adenocarcinoma		Mantle cell lymphoma
	Mucinous adenocarcinoma		Diffuse large B-cell lymphoma
	Signet-ring cell carcinoma		Burkitt lymphoma
	Small cell carcinoma		Burkitt-like /atypical Burkitt-lymphoma
	Squamous cell carcinoma		Others
	Adenosquamous carcinoma		
	Medullary carcinoma		
	Undifferentiated carcinoma	**Secondary tumours**	
		Polyps	
Carcinoid (well differentiated endocrine neoplasm)			
	EC-cell, serotonin-producing neoplasm	Hyperplastic (metaplastic)	
	L-cell, glucagon-like peptide and PP/PYY producing tumour	Peutz-Jeghers	
	Others	Juvenile	
	Mixed carcinoid-adenocarcinoma		
	Others		

1.3. Environmental and genetic risk factors

Development and progression of CRC could be influenced by several risk factors, namely, genetic and environmental factors. These factors include advanced age; family and medical histories of benign adenomatous polyps and inflammatory bowel diseases; low vegetables and fruits intake; high red and processed meat intake[25]; smoking habits; excessive alcohol consumption; physical inactivity; obesity[26]; and diabetes.[1,3,19,24] Furthermore, genetic alterations in tumour suppressor genes and oncogenes that are responsible for MSI; defects in the DNA mismatch repair; mutations; and SNPs in genes involved in the metabolic pathways also have been implicated in CRC.[40] Individual genetic variants arrangements could affect sporadic CRC associated risk when combined with others environmental factors, especially with diet patterns.[41]

Diets with high fat-animal ingestions can influence highly carcinogenesis initial phases, appearing to be an important risk factor for CRC.[6] Genome-wide association studies (GWAS) have identified SNPs in genes involved in the regulation of lipid biosynthesis, transport, and metabolism[42], being some potentials susceptibility factors to CRC. SNPs are mutations with minor allele frequency of greater than 1% in at least one population.[43] Those mutations are characterized by single nucleotide base pair insertions, deletion, or substitutions. SNPs location is also important, if SNP is in an intron, it may change the binding ability of transcription factors to the SNP area, thus influencing gene expression. On the other hand, if the SNP is located in the exon coding region it may affect the structure of the translated protein, which may affect its sensitivity to upstream signal or effectiveness in downstream function.[40] Interestingly, some SNPs are associated with specific ethnic groups or geographic ancestral subpopulations. Effects of lipid metabolism genes polymorphisms associated with CRC risk can be mediated and modified through their implication in energy balance, dietary patterns, obesity or physical activity.[44]

1.4. Lipid metabolism genes and CRC

Cancer is characterized by uncontrolled cell growth with increased proliferation, decreased apoptosis and improves migrating behavior of cells by promoting their ability to invade adjacent tissues and/or metastasize to non-adjacent organs and tissues. Cell proliferation requires duplication of all macromolecular components during each cell division.[45] Aberrant lipid metabolism is a key feature of cancer cells development since cell proliferation requires increased lipid biosynthesis, and lipid catabolism produces bioactive molecules that act as signal molecules to regulate cancer metastasis.[45] Lipids are hydrophobic or amphipathic small molecules, which are insoluble in water but soluble in nonpolar solvents. These molecules form plasma membranes enabling the maintenance of intracellular biological events and the response to changes in the extracellular medium. In living cells, lipids play essential functions, allowing the support of cellular structure, energy supply and participation in cell signaling.[46] Lipid metabolism involves the activation and regulation of multiple signaling pathways. Thus, it enables various connection signaling pathways involved in cell growth regulation, proliferation, differentiation, survival, apoptosis, inflammation, motility and membrane homeostasis. In addition, it can change membrane composition and permeability causing disease development and progression, namely CRC. Three classical lipids (fatty acids, phospholipids and cholesterol) are dramatically increased and actively biosynthesized in cancer cells. Several evidences show that fatty acid synthase expression and activity are extremely low in nearly all nonmalignant adult tissues, whereas it is significantly up-regulated in solid cancers.[45]

Cholesterol is an essential constituent of cell membranes. It is the most frequent steroid in the organism, playing a physiological role. Cholesterol and triglycerides are insoluble in water molecules and are transported in association with proteins. Lipoproteins are complex particles with a central core containing cholesterol esters and triglycerides, a second layer is composed by free cholesterol and phospholipids delimited by apolipoproteins (Figure 3). Plasma lipoproteins are classified into seven classes which are based on their size, lipid composition and apolipoprotein: chylomicrons (CM) and the remaining chylomicrons, VLDL (very low density lipoprotein), IDL (Intermediary density

lipoprotein), LDL (Low Density Lipoprotein), HDL (High Density Lipoprotein). VLDL, IDL and LDL are pro-atherogenic, while HDL is anti-atherogenic.[47]

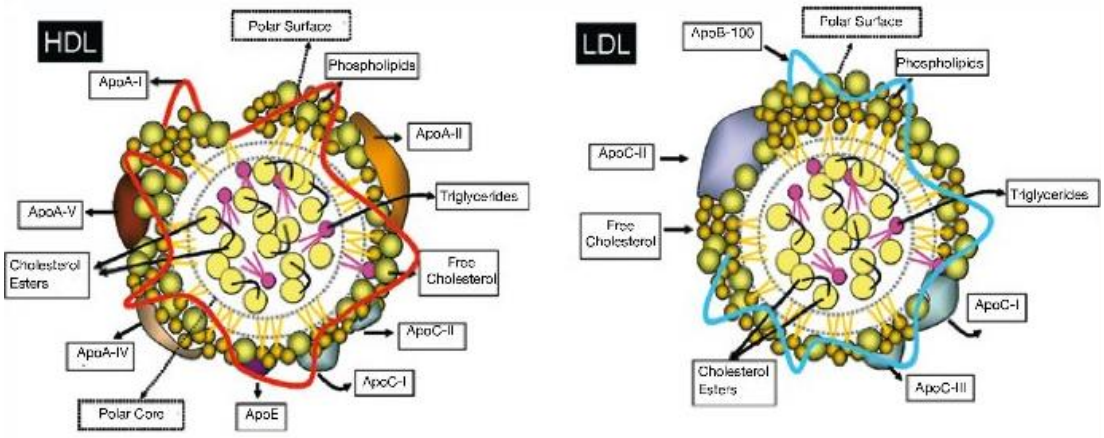

Figure 3. Schematic of high density lipoproteins (HDL) and low density lipoproteins (LDL) structure. Apo indicates apolipoprotein.[48]

Lipoproteins metabolism is divided in three pathways: exogenous diet associated (Figure 4); endogenous liver associated (Figure 5); and reverse cholesterol transport pathway (Figure 6).[49] Lipoproteins play a central role in intake-lipid absorption and transport through the small intestine; in lipids transport from liver to peripheral tissues; and in lipids transport from peripheral tissues to liver and intestine (reverse transport). Exogenous pathway (Figure 4) begins with diet-lipids incorporation into chylomicrons on intestine. Thru circulation, triglyceride are transported in chylomicrons into muscles and adipose tissue here lipoprotein lipase (LPL) metabolize them forming chylomicron remnants, used as energy storage. Cholesterol (chylomicron remnants) is then carried into liver (where it can be used for VLDL and bile acids formation), or back to intestine through bile secretion.[47]

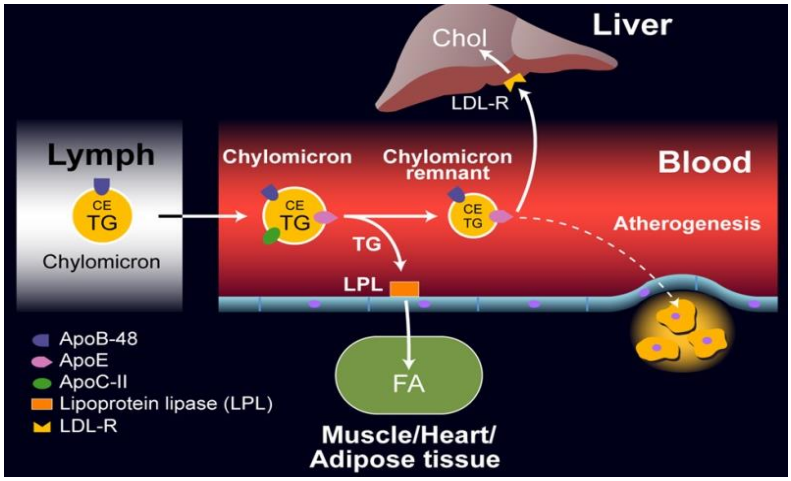

Figure 4. Schematic representation of exogenous cholesterol transport pathway.[47]

Endogenous pathway (Figure 5) begins at liver with VLDL formation. Triglycerides included in VLDL are metabolized and transported into muscle and adipose tissue. In circulation, lipoprotein lipase transforms VLDL into IDL, which is metabolized into LDL. LDL is then absorbed by its receptor in several tissues, including liver, being the predominant uptake site.[47]

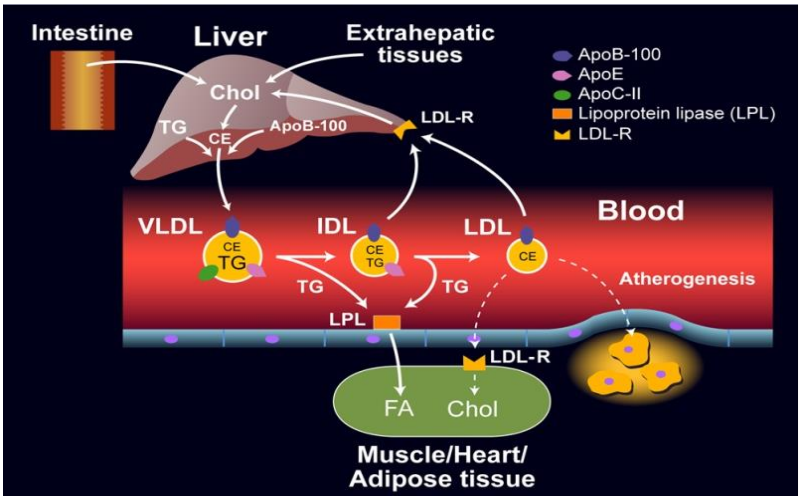

Figure 5. Schematic representation of endogenous cholesterol transport pathway.[47]

Finally, reverse cholesterol transport (Figure 6) begins with HDL formation on liver and intestine. The small particles can acquire HDL cholesterol and phospholipids which are expelled out of cells, a process mediated by ATP-binding cassette transporter A1 (ABCA1) resulting in mature HDL formation. Mature HDL can get cholesterol from cells bias ATP-

binding cassette transporter G1 (ABCG1), scavenger receptor class B1 (SR-B1), or passive diffusion. Then HDL transports cholesterol directly into liver through interaction with hepatic SR-B1; or indirectly through VLDL or LDL cholesterol transfer by a process that is facilitated by CETP. [47]

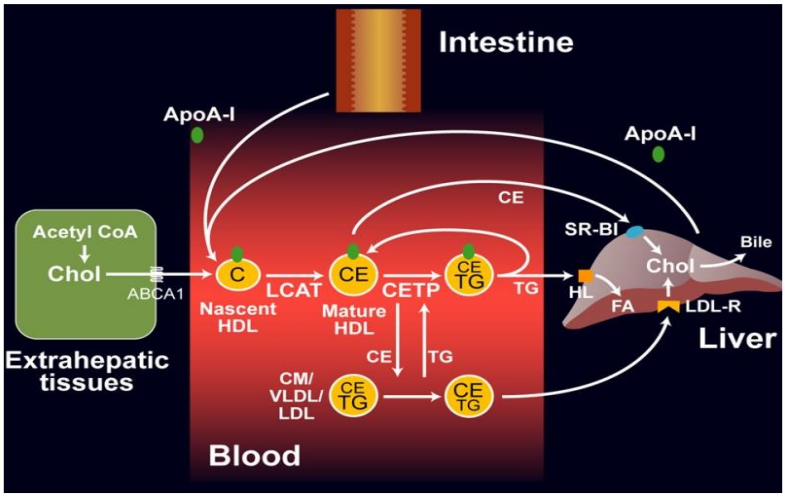

Figure 6. Schematic representation of reverse cholesterol transport pathway.[47]

Apolipoproteins play a structural level function; they act as ligands for receptors and drive lipoproteins formation. In addition to those functions, apolipoproteins also activate and inhibit lipoprotein metabolism enzymes. Thus, they control lipoprotein transport and metabolism, and their dysregulation has been identified in several pathologies. Major apolipoproteins involved in lipid metabolism are APOA1, APOB, APOC3 and APOE.[50] In addition to these proteins, also CETP[51], NPY[52], PON1[53] and PPARG[54] proteins play an essential role in lipid transport and metabolism. As described in the literature genes encoding these proteins contains polymorphic variant associated with various metabolic diseases.[55,56,57,58,59,60,61,62,63]

1.4.1. Apolipoprotein A (APOA1)

Apolipoprotein A1 (APOA1) is a 28-kD protein with 243 amino acid residues, being considered the major surface apoprotein of HDL-C in plasma.[64,65] This molecule is produced primarily in liver (80%) and small intestine (10%)[66], and includes more than 30%

of HDL mass and 70% of HDL protein.[67] APOA1 functions as a cofactor of lecithin-cholesterol acyltransferase (LCAT) and it is a fundamental element of reverse cholesterol transport process.[68] Several studies have shown that the *APOA1* gene displays a key role in tumour progress, inflammation, angiogenesis, invasion and metastasis.[69] The increased APOA1 levels in patients with adenocarcinomas were associated with tumour progression and lymphatic invasion.[70] According to recent findings APOA1 may be considered as a potential diagnosis and prognostics marker in several cancer types.[69]

APOA1 gene located on chromosome 11p23.24 (Figure 7) and have a common polymorphism in promotor region, *APOA1* -75 G>A (rs670).[71,72] In this variant occurs the substitution of guanine (G) by adenine (A), that is associated with HDL-C concentrations and raised serum APOA1. This particular polymorphism occurs with an allele frequency of about 30% in the population general, being 15-20% among Caucasian population.[73] Thus, *APOA1* -75A individuals have higher levels this molecule and/or HDL cholesterol than those that have the wild type allele.[65,74] *APOA1* -75 promoter changes the answers of small and large HDL particle distribution to exercise training.[67] Some recent studies reported and association between different HDL plasma levels and cancer susceptibility, including CRC.[75,76]

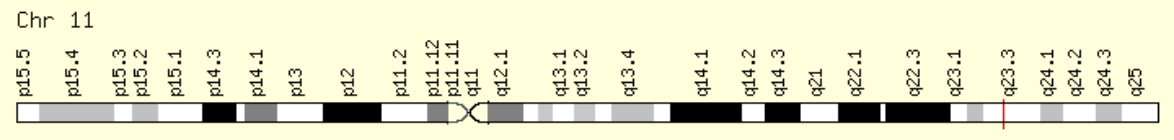

Figure 7. Cytogenetic localization of *APOA1* gene. (Adapted from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=APOA1&keywords=APOA1)

1.4.2. Apolipoprotein B (APOB)

Apolipoprotein B (APOB) is a key protein in the lipid transport and metabolism, such as plasma triglyceride (TG) and cholesterol.[77] Moreover, it is the main apolipoprotein of chylomicrons, as well as VLDL, IDL, LDL particles.[78] APOB is used as ligand for LDL recognition and catabolism, maintaining LDL particles integrity and LDL-C plasma levels control through receptors binding.[79] APOB is produced primarily in hepatocytes and enterocytes. This molecule is present in lipoprotein particles and it presents two isoforms

in humans, APOB-100 and APOB-48. APOB-100, full-length protein containing 4536 amino acids residues, being an indispensable structural protein for VLDL formation and secretion, APOB-100 is expressed primarily in liver.[77] The interactions among dietary and *APOB* genetic variants could affect individual's plasma lipid levels and body weight.[80]

APOB gene is located on short arm of chromosome 2p24.1 (Figure 8), and is composed by 28 introns and 29 exons.[81] Some studies demonstrate an association between the *APOB* SNPs and hypercholesterolemia in Spanish[56] and Hungarian[82] subjects. It has been reported that point mutations in *APOB* receptor-binding zone can disrupt binding and impair LDL removal from circulating. LDL receptor pathway regulates cholesterol plasma concentration. Thus, defects that occur in ligand (APOB) or receptor (LDL-R) could increase LDL plasma concentrations, resulting from inefficient clearance of LDL particles by its receptor. Consequently, it can lead to LDL particles accumulation in circulation that could increase the risk of pathologies related to cholesterol imbalance. An example of these mutations, is the *APOB* R3500Q variant (rs5742904)[83], characterized by a G to A nucleotide transition, in exon 6 of *APOB* gene which origin an amino acid substitution (glutamine for arginine) at position 3500. [81,84,85] In Caucasian population the mutation frequency is very low, 1:500 to 1:700, but its frequency is much higher in several cholesterol dependent disorders, including cardiovascular and cancer diseases.[86]

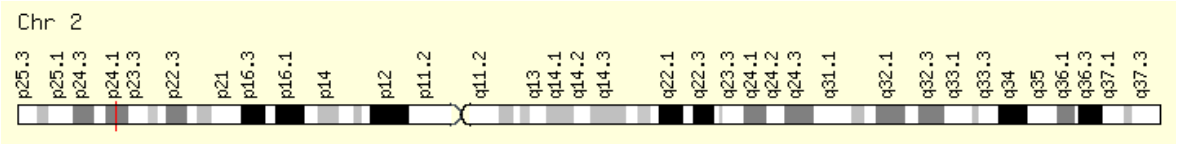

Figure 8. Cytogenetic localization of *APOB* gene. (Adapted from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=APOB&keywords=APOB)

1.4.3. Apolipoprotein C3 (APOC3)

Apolipoprotein C3 (APOC3) is a major component of VLDL and chylomicrons, and it is associated with triglyceride rich lipoproteins (TRLs) levels.[87] This molecule is synthesized by the liver and intestine.[88] APOC3 is an inhibitor of lipoprotein lipase and decreases liver´s absorption of TRL and its remnants (Figure 9). Several studies have

demonstrated that an increase of APOC3 plasma levels is strongly correlated with high triglycerides blood levels.[57]

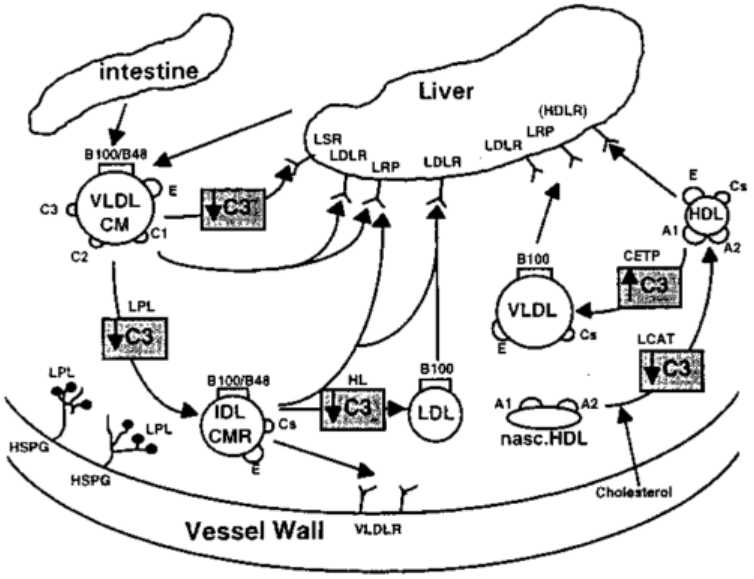

Figure 9. Representation APOC3 effects in lipoprotein metabolism pathways. APOC3 can modulate enzymes that are involved in cholesterol transport from extrahepatic tissues to the liver. APOC3 inhibits (↓) LCAT activity, VLDL TGs clearance and TRLs lipolysis. Furthermore, APOC3 also stimulates (↑) CETP activity[88]

APOC3 gene is located in the long arm of chromosome 11q23 (Figure 10). This gene is about 311kb length and it has 4 exons and 3 introns. Two common polymorphisms in *APOC3* have been reported, C3175G (rs5128) and T3206G (rs4225), characterized by a C to G and T to G substitution, respectively. [57,89] A study performed in Han Chinese males of *APOC3* C3175G variant demonstrated that triglyceride plasma level on mutant homozygous (GG genotype) carriers were higher than *APOC3* 3175CG genotype carriers and *APOC3* 3175CG genotype has higher triglyceride plasma levels than CC genotypes (wild-type). These results suggest that G *APOC3* form may influence TG lipase synthesis. Although 3175G variant is associated with total cholesterol and LDL levels increase, *APOC3* 3206G mutant allele seems to have an inverse effect. A study demonstrated that *APOC3* 3175G mutation allele represents a risk factor and *APOC3* 3206G variant is a benefic factor for cardiovascular diseases, since it is associated with triglycerides blood levels reduction. [57] Nevertheless their role in CRC is not clear.

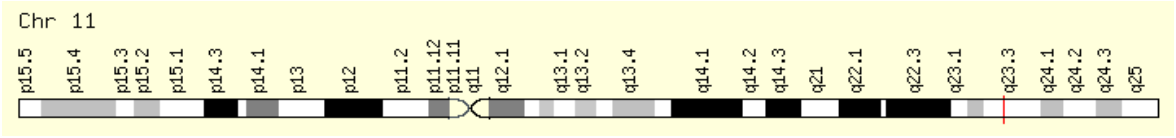

Figure 10. Cytogenetic localization of *APOC3* gene. (Adapted from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=APOC3&keywords=APOC3)

1.4.4. Apolipoprotein E (APOE)

Apolipoprotein (APOE) is a glycoprotein with a molecular mass of 34 kDa of 299 amino acids.[90] This molecule is crucial for cholesterol transport, lipid metabolism and synthesis.[91] In addition, this protein has shown to have an important role in lipid metabolism, angiogenesis, tumour cell growth, metastasis, antioxidant and immune activities.[41] Thus, all these features make APOE a potential candidate in carcinogenesis development. Also, it has a significant metabolic function in the transport and lipids deliver from one tissue or cell type to another, it is synthesized and secreted by many tissues, liver, small intestine (only secrete), brain, skin, and tissue macrophages throughout the body. Furthermore, APOE is a component of VLDL particles.[90]

APOE gene is localized on long arm of chromosome 19q13.2 (Figure 11) and is constituted for four exons and three introns.[92] Some studies had showed that *APOE* exhibit multiple isoforms: E2, E3 and E4. Those variants combine in six different genotypes: three homozygous phenotypes (E2/2, E3/3, and E4/4) and three heterozygous phenotypes (E3/2, E4/2, and E4/3).[90] All alleles are different in primary structure at two sites: residues 112 (rs429358) and 158 (rs7412).[41] *APOE*3* has a cysteine at residue 112 and arginine at residue 158, while *APOE*4 has 2 arginine and *APOE*2 has 2 cysteine in both 158/112 residues. APOE*2 and APOE*4 is associated with increased and decrease APOE levels, respectively.[90] The presence of different *APOE* variants influences the enterohepatic metabolism of cholesterol and bile acids, which promote colorectal carcinogenesis.[58,93,94] A study carried in Finland showed that the presence of *APOE* E4 allele could provide protection from adenoma and carcinoma development on proximal colon, due to reduced bile acids production.[93] On the other hand, some studies demonstrate that women with high triglycerides levels and with one or two copies of

APOE E4 allele had four times higher risk of developing breast cancer when compared with women with low triglyceride levels.[91,95]Nevertheless, E2, E3 and E4 isoforms are associated with several cancers predisposition[96,97], including colorectal cancer[93], being *APOE* E3 allele the most predominant form[98].

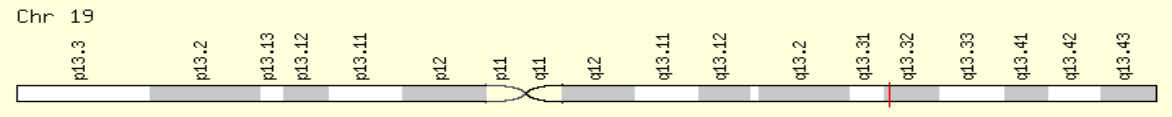

Figure 11. Cytogenetic localization of *APOE* gene. (Adapted from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=APOE&keywords=APOE)

1.4.5. Cholesterol ester transfer protein (CETP)

Cholesterol ester transfer protein (CETP) has an important role in HDL plasma levels regulation. CETP helps in cholesteryl ester and triglyceride transfer and exchange between HDL, LDL and VLDL. Moreover, it also plays a role in liver's cholesterol ester uptake and in plasma lipoproteins quantity and quality control.[51,99]

CETP gene is composed by 16 exons[59] and is located in 16q13 chromosome (Figure 12). Findings have described two genetic variants, involved in several diseases, *CETP* G279A (rs708272) and *CETP* R451Q (rs1800777).[100] *CETP* G279A variant has been identified as an essential cholesterol concentration regulator within HDL fraction. *CETP* 279G allele (allele B1) is responsible for higher CETP activity and lower cholesterol concentration in HDL fraction. While, the presence of *CETP* 279A allele (allele B2) is related with lower CETP activity and higher HDL values.[60] On other hand, there is R451Q mutation, in which occurs a G to A substitution, modifying arginine (451) to glutamine in CETP protein.[99] *CETP* 451Q mutant allele appears in low frequency in general population, about 2-7%, and has been associated with higher CETP activity and lower HDL-C levels. [59,101]

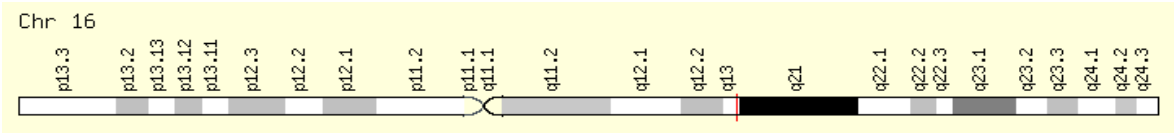

Figure 12. Cytogenetic localization of *CETP* gene. (Adapted from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=CETP&keywords=CETP)

1.4.6. Neuropeptide Y (NPY)

Neuropeptide Y (NPY) is a neurotransmitter present in central and peripheral nervous system, several peripheral organs, and plasma. This plays an important role in glucose-stimulated insulin secretion inhibition, renal blood flow regulation and is implicated in control and management feeding behaviors and food intake.[102] NPY has the ability to induce obesity; this propriety was already confirmed in animal models.[103] This fact, results in an increase of plasma insulin concentration. Findings indicate that *NPY* gene is a prime candidate gene for obesity.[104] Moreover, also have been demonstrated that NPY promotes proliferation and vascularization, stimulate migration, formation of capillary tubes, development of blood vessels and it has many immunomodulatory functions, including modulates of the functions of macrophages.[105,106]

NPY gene is located in chromosome 7p15.1 (Figure 13) and has a SNP, *NPY* Leu7Pro (rs16139)[107], associated with *NPY* process, levels growth hormone secretion and serum cholesterol and triglyceride levels. In the *NPY* Leu7Pro SNP occurs a thymidine to cytosine nucleotide substitution (T1128C) in a coding region of *NPY* gene, interfering with signal pathway.[102] This mutation is linked with the increase of the *NPY* synthesis and secretion levels being more frequent in Caucasian population (6% to 15%).[106] Also, higher serum total cholesterol and lipid levels, and increased body mass index (BMI) are related with the *NPY* Leu7Pro polymorphism.[61,108,109,110] Nevertheless, its implication on CRC risk is not yet clarified, although there is a strong association between NPY and obesity, other cancer types and colorectal predisposal.[111,112]

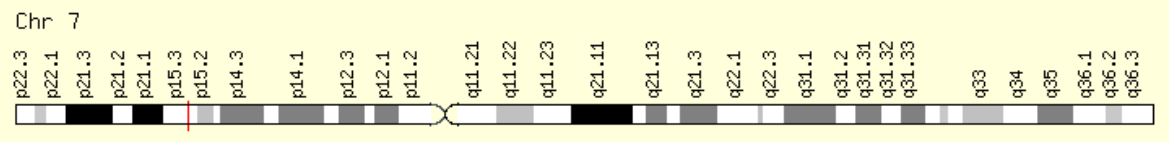

Figure 13. Cytogenetic localization of *NPY* gene. (Adapted from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=NPY&keywords=NPY)

1.4.7. Paraoxonase 1 (PON1)

Paraoxonase 1 (PON1) is a protein with 354 amino acids and a molecular mass of 43 KDa. This enzyme is synthesized mainly in liver and secreted into blood, where it is associated exclusively with HDL (Figure 14).[113,114] In animal models and human HDLs are major carriers of plasma lipid hydroperoxides (LOOHs). Moreover, PON1 enzyme has several roles such as protection against oxidative damage and lipid peroxidation, helps in the innate immunity, reactive molecules detoxification, drugs bioactivation, endoplasmic reticulum stress modulation and cell proliferation/ apoptosis regulation.[115] Some studies confirmed that PON1 prevents oxidized LDL formation and inactivates LDL-derived oxidized phospholipids once they are formed; furthermore it also protects phospholipids in HDL from oxidation.[116] A diet rich in fat and cholesterol modulate *PON1* activity.[117]

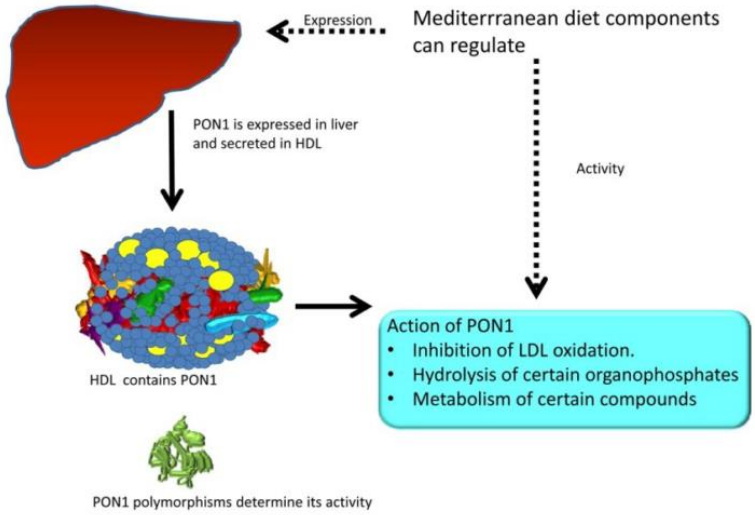

Figure 14. Effect of dietary components on regulation of PON1 activity and its interaction with genetic factors.[117]

PON1 gene is located in chromosome 7q21.3 (Figure 15) and is coded by nine exons. This gene has a polymorphism in the coding region, involved in various diseases and conditions, *PON1* Q192R (rs662;A>G), resulting in Glutamine (Q) to Arginine (R) substitution 192 codon, leading to differences in hydrolytic activity towards paraoxon.[113] Like other genes ethnicity and diseases can affect its distribution frequencies. Patients with CRC exhibit low PON1 plasma, high lipid peroxide and LOOH levels. This suggests a possible involvement of oxidative stress and *PON1* variants in CRC.[118,119,62,120]

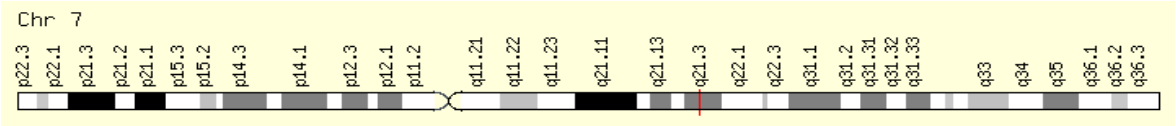

Figure 15. Cytogenetic localization of *PON1* gene. (Adapted from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=PON&keywords=PON)

1.4.8. Peroxisome proliferator-activated receptor gamma (PPARG)

Peroxisome proliferator-activated receptor gamma (PPARG) belongs to a group of nuclear hormone receptor that behaves as transcription factors that regulate genes expression. When PPARG interacts with the specific ligands is translocated into the nucleus, changes its structure and regulates gene transcription. PPARG plays an essential role in lipid metabolism, adipocytes differentiation and immune regulation. There are three isoforms of PPARG occurring by alternative splicing and promotors: PPARG1; PPARG2; and PPARG3.[121]

PPARG gene is located in 3p25.1 chromosome (Figure 16). A common variant has been found in *PPARG*, characterized by a C to G substitution at exon 2. This alteration change Proline to Alanine at codon 12 (Pro12Ala) of *PPARG* gene, which reduces its promoter affinity and transcriptional activity. Some studies have shown that *PPARG* loss-of-function is associated with the increased risk of CRC.[63,122] High activity of *PPARG* prevents the S-phase entry into the cell cycle, inhibiting the proliferation of malignant cells. It also has been shown that the nuclear factor kappa B (NF-B) and signal transducer and activator of transcription 3 (STAT3) inflammation pathways and cell growth could be inhibited by ligand activation of PPARG, allowing apoptosis and promote differentiation in

colon, breast and prostate cell lines.[121,123] However, other studies carried out in mice with a high fat diet, shows that the activation of *PPARG* is associated with the CRC progression.[124]

PPARG Pro12Ala (rs1801282)[125] substitution is associated with reduced transactivation activity. High activation of *PPARG* can lead to development of tumours by inhibition of expression of prostanoid and integrin receptors, by reducing the expression of fibronectin and by inhibiting angiogenic production and inflammatory signals. Furthermore, PPARG ligands may increase tumour suppressors, such as PTEN (Phosphatase and tensin homolog) and p21, expression and/or activity.[125] Some works suggest that the presence of the *PPARG* 12Ala allele, which is associated with reduced PPARG activity, might decrease colon cancer susceptibility.[126] *PPARG* 12Ala was recognized to be associated with increased tissue sensitivity to insulin, a decrease in insulin plasma level, reduced release of free fatty acids by adipocytes, which may indirectly explain the lower colon cancer risk in subjects with this polymorphism.[125] Nevertheless its role in rectum cancer is not entirely clarified, seeming to have an opposite role in this type of tissue.

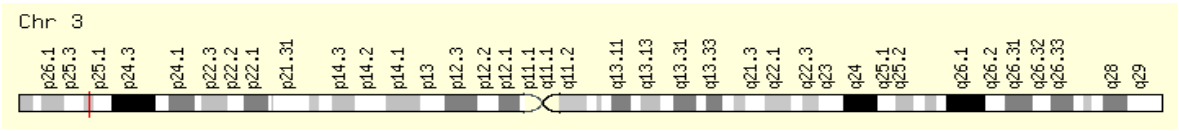

Figure 16. Cytogenetic localization of *PPARG* gene. (Adapted from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=PPARG&keywords=PPARG)

1.5. Lipid transport and metabolism genes in Sporadic Colorectal Cancer (SCA)

Gene variants associated with various environmental and dietary factors can affect sporadic colorectal cancer risk. High fat diets are a fundamental risk factor for CRC development[127], and its influence appears to be stronger during post-initiation phases of carcinogenesis.[6] Accordingly, molecular mechanisms and pathways that could interfere in lipid transport and metabolism should be considered as a potential risk modulators for this neoplasia. Multiple genetic determinants, comprising several gene polymorphisms,

involved in lipid metabolism regulation, as well as, in lipid transport had been reported as risk factors for CRC.[8] *APOA1*, *APOB*, *APOC3*, *APOE*, *CETP*, *NPY*, *PPARG* and *PON1* genes have a considerable role in lipid transport regulation and metabolism, therefore numerous studies which including their common polymorphic variants demonstrate that they could affect these pathways.[55,56,57,58,59,60,61,62,63] However, for most of the studied SNPs is unowned their association and influence on CRC.

Most authors recognize that a high fat diet is essential to raise the CRC risk by increasing bile acids secretion.[128,129,130] This increased secretion has a non-specific irritant effect on colonic and rectal mucosa since it stimulates secondary bile acids formation. This effect can cause damage in intestinal epithelium bias oxidative stress and lipid peroxidation, stimulating endogenous mutations and increasing tumour proliferation. [128,129,130,131]

Since *APOA1*, *APOB*, *APOC3*, *APOE*, *CETP*, *NPY*, *PPARG* and *PON1* polymorphisms are responsible for different enzymatic activities among individuals, that consequently control the bile acids metabolism, and also because the role of these polymorphisms in SCA pathological progression has not been fully explained. Our study aims to clarify these issues by coming across the relationship between *APOA1*, *APOB*, *APOC3*, *APOE*, *CETP*, *NPY*, *PPARG* and *PON1* genotypes and SCA, as well as their interaction with other molecules.

Chapter II
Material and Methods

2. Materials and methods

2.1. Biological samples

This study included 68 SCA biopsies samples formalin-fixed and paraffin-embedded (FFPE) (mean age of 73.7 +/-10.7 years; 75% men and 25% women), previously selected. As control group were used 100 biopsies of healthy subjects, from screening (mean age of 72.7 +/-8.9 years; 79% men and 21% women) (Table 3). Biopsies were collected from 2009 to 2011 from the archives of Institute of Anatomical Pathology, Faculty of Medicine of the University of Coimbra. SCA samples were clinically identified as belonging to colon (31 samples) and rectum (37 samples) carcinomas and were selected according to the malignant cells availability with at least 60 malignant cells. SCA cases were classified according to WHO criteria[39] (Table 2), being all samples used here classified as well differentiated adenocarcinoma (Figure 17). This study was supported and approved by local ethics committee (CIMAGO - Faculty of Medicine of the University of Coimbra, Coimbra, Portugal).

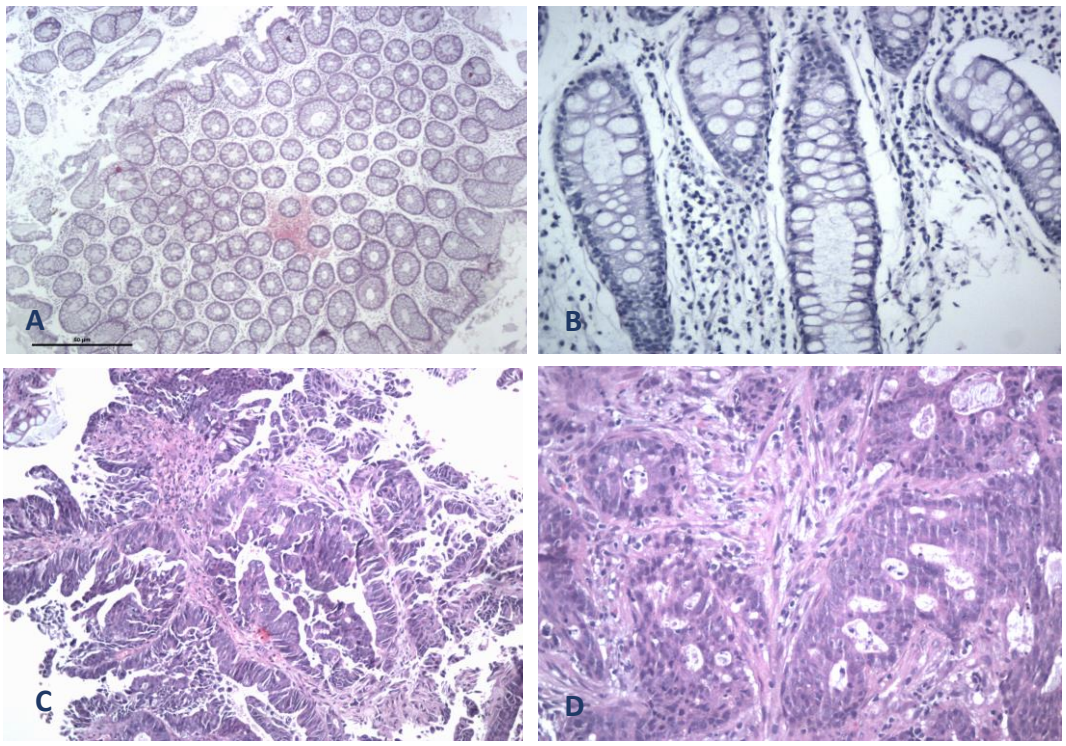

Figure 17. (A), **(B)** Histologic image of normal colonic mucosa: 40x HE (Haematoxylin-eosin), 200x HE; **(C)**, **(D)** Histological image of well differentiated sporadic colorectal adenocarcinoma (According to WHO criteria). 100x HE; 200x HE. Images from IAP-FMUC.

Table 3. Clinical pathological data SCA and controls.

					Tumour localization				
		Distribution	Age		Colon		Rectum		Total
	Gender	%	Mean	Stand. Dev.	n	%	n	%	
SCA	Male	75	73.7	10.7	25	81	26	70	51
	Female	25			6	19	11	30	17
	Total	100			31	100	37	100	68
Controls	Male	79	72.7	8.9					
	Female	21							
	Total	100							

2.2. DNA extraction

DNA genomic isolation from biopsies were made according to the extraction protocol from *NZY Tissue gDNA Isolation Kit* (NZYTech, Lisbon, Portugal), after microdissection of the normal and tumour tissue (5 to 10 dissections of 10µm of thickness to each sample). Samples were prepared by adding 1 ml of xylene to each tube to paraffin removal. After centrifugation at 11,000x*g* for 3 min, supernatant was discarded and samples were washed with 1ml of ethanol (96%-100%), repeating the centrifugation step. Next to this process, cellular membrane lysis were done by adding 25 µl of Proteinase K and 180 µl of Buffer NT1 into each sample, and mixed thoroughly by vortex and incubated at 56 ˚C overnight. Afterwards, 210 µl of ethanol (96%-100%) was load to the samples, and mixed immediately by vortex. In a next step occurred the DNA binding and the mixture was transferred to into a NZYSpin Tissue Column placed in a 2 ml collection tube, and centrifuged for 1 min at > 11,000x*g*. After this, the flows-through were discarded and the columns were placed back into a new collection tube. Washing silica membrane steps are essential to impurities elimination and were done by adding 500 µL of Buffer NW1 to the columns, centrifuged (for 1 min at > 11,000x*g*) and the flows-through were discarded. After this, the same procedure was repeated using 600 µL of Buffer NW2. Then, to dry the silica membrane the column was centrifuged for 2 min at > 11,000*xg.* Finally, the columns were placed into a clean microcentrifuge tube and add 100 µl of sterile water, incubated 1 min at room temperature and centrifuged at > 11,000x*g* for 2 min to elute DNAs.

Genomic DNA samples were stored at -20 ˚C following of purity and concentration determination of all DNA samples, by spectrophotometry, applying a wavelength of 260/280nm.

2.2.1. Analysis of concentration and quality of the extracted DNA

DNA samples were quantified in a spectrophotometer *GeneQuant pro* (Biochrom, Cambridge, England). Rnase-free water was applied as reference and 7 µl of DNA sample were inserted in the ultra-microvolume *cuvette* in order to perform the sample concentration and purity quantification, by reading adequate optical densities (230 nm, 260 nm and 280 nm). The presence of nucleotides and proteins were detected at a wavelength of 280 nm, while at the 260 nm wavelength only detects nucleotides. At a wavelength of 230 nm, the presence of contaminants was assessed.

2.3. Genotyping

Sample genotyping was performed by PCR-SSP (*Polymerase Chain Reaction – Sequence Specific Primers*) technique, based on the specific amplification of defined regions allele. Several polymorphic points of genes encoding: *APOA1* -75 G>A*, APOB* R3500Q*, APOC3* C3175G*, APOC3* T3206G*, APOE* Cys112Arg*, APOE* Arg158Cys*, CETP* G279A*, CETP* R451Q*, PON1* Q192R*, NPY* Leu7Pro*, PPARG* Pro12Ala (Table 4). Primers used were obtained by collaboration with Genebox (Biocant- Cantanhede, Portugal) "Nutri Box Kit".

Table 4. Lipid metabolism common polymorphisms analyzed in this study.

Gene	Genetic variant	NCBI SNP DataBase	Region of gene	Cytogenetic Localization	Position	Alleles
APOA1	*APOA1* -75 G>A	rs670	Promotor	11p23.24	-75	G>A
APOB	*APOB* R3500Q	rs5742904	Exon 26	2p24.1	3500	G>A
APOC3	*APOC3* C3175G *APOC3* T3206G	rs5128 rs4225	Exon 4 3'UTR	11q23	3175 3206	C>G T>G
APOE	*APOE* Cys112Arg *APOE* Arg158Cys	rs429358 rs7412	Exon 4	16q13.2	112 158	C>T
CETP	*CETP* G279A (TaqIB) *CETP* R451Q (G1533A)	rs708272 rs1800777	Intron 1 Exon 15	19q13.32	279 1533	G>A G>A
NPY	*NPY* Leu7Pro (T1128C)	rs16139	Coding region	7q15.1	1128	T>C
PON1	*PON1* Q192R	rs662	coding region	7p21.3	192	A>G
PPARG	*PPARG* Pro12Ala	rs1801282	Exon 2	3p25.2	12	C>G

2.3.1. Amplification by *Polymerase Chain Reaction-Sequence Specific Primers* (PCR-SSP)

Polymorphisms genotyping was carried through commercial kits "Nutri Box Kit" (Genebox, Cantanhede - Portugal) using PCR-SSP (*Polymerase Chain Reaction – Sequence Specific Primers*) technique. These kits included internal, negative and positive controls for each sample. In this process it was used for typing plates 96 wells, and was placed into each well 5 µL of the pair of specific primers, 2 µL of DNA sample and 3 µL of reaction mixture. The plates were sealed and placed in a MyCycler Thermal Cycler of 96 wells (Biorad, California, USA). The detection of mutations was performed using the displayed program present in Table 5. The amplified PCR products was analysed by electrophoresis with a SYBR Safe (Molecular Probes, Oregon – USA) in 2% agarose gel and visualized in a ultra-violet (UV) transilluminator (UVi Tech, Cambridge, United Kingdom).

Table 5. Protocol of the program to amplification by PCR-SSP (*Polymerase Chain Reaction – Sequence Specific Primers*), from Genebox, Cantanhede – Portugal.

Step	Temperature	Time	Number of cycles
Initial denature	95º C	1 Min	1
Denature	95º C	25 Sec	
Annealing	70º C	45 Sec	5
Extension	72º C	30 Sec	
Denature	95º C	25 Sec	
Annealing	65º C	45 Sec	21
Extension	72º C	30 Sec	
Denature	95º C	25 Sec	
Annealing	55º C	1 Min	4
Extension	72º C	2 Min	
Final extension	72º C	10 Min	1

2.3.2. Electrophoresis in agarose gel

PCR reactions, after amplification, were submitted to electrophoresis by 2% agarose gel in order to identify the amplified products. Agarose *Routine Grade* (NZYTech, Lisbon, Portugal) was dissolved in 1x TAE (Tris-acetate-EDTA) (NZYTech, Lisbon, Portugal) and distillated water and agitated for 15 seconds. Then the solution was transferred to the microwaves to complete dissolution of agarose (approximately 2 min at 900w). Afterwards, the solution was cool down and $1x10^{-5}$ SYBR Safe (Molecular Probes, Oregon – USA) a dye that allows the visualization of DNA under the UV light incidence, were added and the solution was agitated for 15 seconds for homogenization. Solution was after putted into a cradle until it solidifies under the environment temperature. After solidification, the solidified gel was inserted in the plastic gel box, previously filled with 1x TAE (NZYTech, Lisbon, Portugal). PCR samples were inserted into the gel wells and they were left running for 10 minutes under the 300 volts of the *Power Pac Basic* device (Bio-

Rad, California, USA). Finally, PCR products were visualized under UV light by a transilluminator (UVi Tech, Cambridge, United Kingdom). Results were further registered by a digital camera (NIKON DMX1200F).

2.4. Statistical analysis

2.4.1. Analysis of purity and concentration of DNA

DNA samples purity and concentration study consisted on the calculation of the means, standard-deviations and confidence levels relatively to their concentrations and contamination quantity. This statistical analysis aimed to verify if the conditions of the DNAs were acceptable for the validation of the results in the study.

2.4.2. Allele and genotype frequencies of polymorphisms

Allele and genotype frequencies were calculated from obtained percentages for each studied polymorphism. In order to assess if the mutations were in equilibrium, *chi*-square test was performed using the Hardy-Weinberg equilibrium as reference. *APOA1* -75 G>A, *APOB* R3500Q, *APOC3* C3175G, *APOC3* T3206G, *APOE* Cys112Arg, *APOE* Arg158Cys, *CETP* G279A, *CETP* R451Q, *NPY* Leu7Pro, *PON1* Q192R and *PPARG* Pro12Ala frequencies were compared between different groups (SCA versus control and tumour localization) using STATISTICA 14 (StatSoft, Inc., 2013) based on *chi*-square (χ^2) test and Exact Fisher test. The significance level was set at $p<0.05$, odds ratio (OR) and 95% confidence intervals (CI) for relative risks (RR) were also calculated for each variation.

Chapter III
Results

3. Results

3.1. Clinical pathology data

The individual distributions of SCA subjects included in the study, according to their clinical pathological features of biological samples are presented in the Table 3. The distribution of SCA subjects by gender shows a predominance of the disease in male subjects comparing with female individuals (75% versus 25%). In terms of tumour localization, it was observed a small difference of gender distribution among colon and rectum groups, however, not significant differences were found (Table 6). Moreover, 46% (31/68) of the SCA biopsies were located in colon and 54% (37/100) of the SCA biopsies were located in rectum (Table 6). Also, there were not significant differences between mean age in both groups (colon samples: mean age of 73.7 +/- 10 years; rectum samples: mean age of 71.2 +/-12.1 years).

Table 6. Gender distribution among colon and rectum groups.

	Colon		Rectum		*p-value*	95% IC	
Gender	**n**	**%**	**n**	**%**		**OR**	**RR**
Male	25	81	26	70	0.364	0.567 (0.182-1.767)	0.788 (0.506-1.226)
Female	6	19	11	30		1.763 (0.566-5.491)	1.269 (0.816-1.975)
Total	31	100	37	100			

3.2. Analysis of DNA purity and concentration

Although DNA concentration were not uniform (+/-29.3μg/ml) and lower than the standard value (100 μg/ml)[132] (Table 7), the amplification by PCR-SSP occurred without problems, since the amplification protocol was adapted to the DNA concentration of samples. Although the mean values of purity of DNAs range between acceptable limits, 1.6-1.8 to O.D.260nm/O.D.280nm[132] and 0.4-0.6 to O.D.230nm/O.D.260nm[132], some DNAs show high quantities of contaminants and proteins with a confidence interval being above certain acceptable limits (Table 7).

Furthermore, coefficients of variation show the existence of samples that deviate from acceptable patterns, whether in DNA concentration and in contaminants quantity. These DNA can affect some results, however the majority of samples show a level of purity highly acceptable (Table 7). Globally, PCR-SSP did not present major amplification problems.

Table 7. Means and standard-deviations of DNA samples purity and concentration values

	Concentration	O.D.260nm/O.D.280nm	O.D.230nm/O.D.260nm
Mean	42.4	1.83	0.54
Standard Deviation	29.316	0.09	0.15
Variation Coefficient	69.14%	5%	28%
Confidence Interval (95%)		1.7-1.9	0.4-0.7

3.3. Analyze of allele and genotype frequencies from polymorphisms in SCA and controls

Allele and genotype frequencies from polymorphisms of *APOA1, APOB, APOC3, APOE, CETP, NPY, PON1* and *PPARG* genes in SCA subjects and controls are exhibited in Tables 8-13. Almost all genes presented statistically significant differences between both groups to allele frequencies, except the *APOE* gene and *APOC3* T3206G variant had not statistically significant differences among groups (Table 8). According to genotype frequencies, most genes exhibited statistically significant difference between different groups, excluding *APOA1* -75GG, *APOA1* -75GA, *APOC3* 3206TT, *APOE* E2/E2, *APOE* E2/E3, *APOE* E2/E4, *APOE* E3/E3, *CETP* 451CG, *PON1* 192GG, *PPARG* 12CC and *PPARG* 12CG that had no statistically significant differences among groups (Table 9, 11 and 13). Finally, most of the polymorphisms analyzed were in Hardy Weinberg equilibrium (p>0.05), except the *APOC3* T3206G (*p*=0.02) and *PON1* Q192R (*p*=0.05) (Table 20).

3.3.1. *APOA1* -75 G>A, *APOB* R3500Q, *APOC3* C3175G, *APOC3* T3206G and *APOE* Cys112/158Arg distribution among SCA and controls

Allele frequencies of *APOA1* -75G (wild-type - wt) polymorphism were significantly higher in controls than SCA subjects (52% versus 69%; *p*=0.002; OR= 0.49; 95% CI 0.31-0.77) (Table 8 and Figure 18.A). In contrast, allele frequencies of *APOA1* -75A (mutant) polymorphism was more prevalent in SCA subjects (48% versus 31%; *p*=0.002; OR=2.04; 95% CI 1.30-3.20). Related to genotype frequencies, *APOA1* -75AA (mutant genotype) was significantly higher in SCA subjects than among controls (32% versus 12%; *p*=0.001; OR=3.51; 95% CI 1.59-7.72) (Table 9 and Figure 18.B).

Allele frequencies of *APOB* 3500G (wt) polymorphism were significantly higher in controls than in SCA subjects (79% versus 99%; *p*<0.0001; OR=0.04; 95% CI 0.01-0.17), while *APOB* 3500A (mutant) polymorphism was more prevalent in SCA subjects (21% versus 1%; *p*<0.0001; OR=25.67; 95% CI 6.00-109.82) (Table 8 and Figure 18.C). *APOB* 3500GG genotype (wt) was significantly higher in controls than among SCA subjects (66% versus 99%; *p*<0.0001; OR=0.02; 95% CI 0.003-0.15) (Table 9 and Figure 18.D). Contrasting, *APOB* 3500GA heterozygotic genotype was more frequent in SCA subjects (27% versus 1%; p<0.0001; OR=35.64; 95% CI 4.62-274.70). Furthermore, the genotype *APOB1* 3500AA was presented only in SCA subjects (7% versus 0%; *p*=0.01).

Allele frequencies of *APOC3* 3175C (wt) polymorphism were significantly higher in controls than in SCA subjects (54% versus 93%; *p*<0.0001; OR=0.09; 95% CI 0.05-0.17), however allele frequencies of *APOC3* 3175G (mutant) polymorphism was more frequent in SCA subjects (46% versus 7%; *p*<0.0001; OR=11.13; 95% CI 5.87-21.10) (Table 8 and Figure 18.E). *APOC3* 3175CC wt genotype was significantly higher in controls than in SCA subjects (28% versus 83%; *p*<0.0001; OR=0.08; 95% CI 0.04-0.17), whereas *APOC3* 3175CG (53% versus 15%; *p*<0.0001; OR=6.38; 95% CI 3.08-13.18) and *APOC3* 3175CC (19% versus 2%; *p*=0.0002; OR=11.58; 95% CI 2.52-53.22) mutant genotypes were more prevalent among SCA subjects (Table 9 and Figure 18.F).

Finally, *APOC3* 3206TG genotype was significantly higher in controls than in SCA subjects (19% versus 37%; *p*=0.02; OR=0.40; 95% CI 0.19-0.83), while genotype *APOC3* 3206GG was present only SCA subjects (19% versus 0%; *p*=0.0001) (Table 9 and Figure

18.G), and *APOE E4/E4* genotype showed significant differences, being present only in controls subjects (0% versus 8%; *p*=0.02) (Table 9 and Figure 18.H).

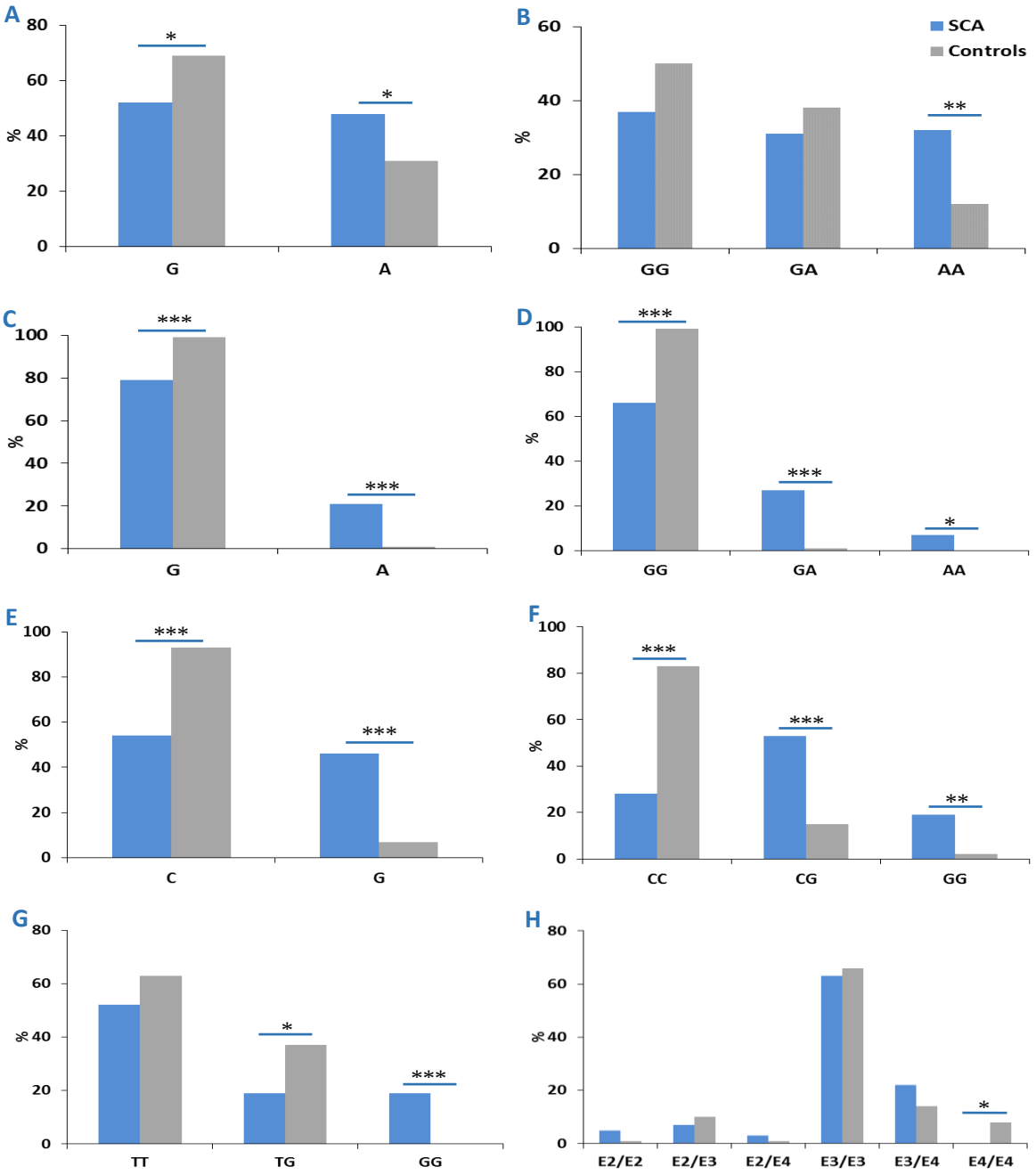

Figure 18. Allele and genotype frequencies of apolipoproteins genes polymorphisms among the SCA and controls subjects: **(A)** Allele frequencies and **(B)** Genotype frequencies of *APOA1* -75 G>A; **(C)** Allele frequencies and **(D)** Genotype frequencies of *APOB* R3500Q; **(E)** Allele frequencies and **(F)** Genotype frequencies of *APOC3* C3175G; Genotype frequencies of **(G)** *APOC3* T3206G and **(H)** *APOE* Cys112/158Arg (* p≤0.05; **p≤0.001; ***p≤0.0001).

Table 8. Allele frequencies distribution of *APOA1* -75 G>A, *APOB* R3500Q, *APOC3* C3175G, *APOC3* T3206G and *APOE* Cys112/158Arg among SCA and control subjects.

Allele Frequencies	SCA and Controls						
	SCA		Controls		*p-value*	95 % CI	
	n	%	n	%		OR	RR
***APOA1* -75 G<A**							
G	71	52	138	69	**0.002***	0.49 (0.31-0.77)	0.66(0.51-0.86)
A	65	48	62	31		2.04 (1.30-3.20)	1.51 (1.17-1.94)
Total	136	100	200	100			
***APOB* R3500Q**							
G	108	79	198	99	**<0.0001***	0.04 (0.01-0.17)	0.38 (0.32-0.45)
A	28	21	2	1		25.67 (6.00-109.82)	2.64 (2.21-3.16)
Total	136	100	200	100			
***APOC3* C3175G**							
C	74	54	186	93	**<0.0001***	0.09 (0.05-0.17)	0.35 (0.28-0.43)
G	62	46	14	7		11.13 (5.87-21.10)	2.87 (2.30-3.57)
Total	136	100	200	100			
***APOC3* T3206G**							
T	97	71	126	63	n.s	1.46 (0.91-2.34)	1.26 (0.94-1.69)
G	39	29	74	37		0.68 (0.43-1.09)	0.79 (0.59-1.07)
Total	136	100	200	100			
***APOE* Cys112/158Arg**							
E2	13	10	13	6		1.52 (0.68-3.39)	1.26 (0.84-1.90)
E3	106	78	156	78	n.s	1.0 (0.59-1.69)	1.0 (0.73-1.36)
E4	17	12	31	16		0.78 (0.41-1.47)	0.86 (0.57-1.29)
Total	136	100	200	100			

*Statistically significant; n.s: not significant

Table 9. Genotype frequencies distribution of *APOA1* -75 G>A, *APOB* R3500Q, *APOC3* C3175G, *APOC3* T3206G and *APOE* Cys112/158Arg among SCA and control subjects.

Genotype Frequencies	SCA and Controls						
	SCA		Controls		*p*-value	95 % CI	
	n	%	n	%		OR	RR
***APOA1* -75 G>A**							
GG	25	37	50	50	n.s	0.58 (0.31-1.09)	0.72 (0.49-1.06)
GA	21	31	38	38		0.73 (0.38-1.40)	0.83 (0.55-1.24)
AA	22	32	12	12	**0.001***	3.51 (1.59-7.72)	1.88 (1.34-2.65)
Total	68	100	100	100			
***APOB* R3500Q**							
GG	45	66	99	99	**<0.0001***	0.02 (0.003-0.15)	0.33 (0.25-0.42)
GA	18	27	1	1		35.64 (4.62-274.70)	2.82 (2.20-3.62)
AA	5	7	0	0	**0.01***	Infinity (NaN-Infinity)	2.59 (2.13-3.14)
Total	68	100	100	100			
***APOC3* C3175G**							
CC	19	28	83	83	**<0.0001***	0.08 (0.04-0.17)	0.25 (0.16-0.39)
CG	36	53	15	15		6.38 (3.08-13.18)	2.58 (1.83-3.64)
GG	13	19	2	2	**0.0002***	11.58 (2.52-53.22)	2.41 (1.80-3.22)
Total	68	100	100	100			
***APOC3* T3206G**							
TT	42	52	63	63	n.s	0.95 (0.50-1.79)	0.97 (0.67-1.41)
TG	13	19	37	37	**0.02**	0.40(0.19-0.83)	0.56 (0.34-0.93)
GG	13	19	0	0	**<0.0001***	Infinity (NaN-Infinity)	2.82 (2.28-3.48)
Total	68	100	100	100			
***APOE* Cys112/158Arg**							
E2/E2	3	5	1	1		4.57 (0.47-44.88)	1.89 (1.04-3.44)
E2/E3	5	7	10	10		0.71 (0.23-2.19)	0.81 (0.39-1.70)
E2/E4	2	3	1	1	n.s	3.0 (0.27-33.76)	1.67 (0.73-3.79)
E3/E3	43	63	66	66		0.89 (0.47-1.69)	0.93 (0.64-1.36)
E3/E4	15	22	14	14		1.74 (0.78-3.89)	1.36 (0.90-2.05)
E4/E4	0	0	8	8	**0.02***	0 (0-NaN)	0 (0-NaN)
Total	68	100	100	100			

*Statistically significant; n.s: not significant

3.3.2. *CETP* G279A and *CETP* R451Q (G>A) polymorphisms distribution among SCA and controls

Allele frequencies *CETP* 279G (wt) polymorphism were significantly higher in controls than in SCA subjects (50% versus 71%; $p<0.0001$; OR=0.41; 95% CI 0.26-0.64), contrarily allele frequencies of *CETP* 279A mutant polymorphism were more prevalent in

SCA subjects (50% versus 29%; *p*<0.0001; OR=2.45; 95% CI 1.55-3.86) (Table 10 and Figure 19.A). *CETP* 279GG wt genotype was significantly higher in controls than among SCA subjects (12% versus 71%; *p*<0.0001; OR=0.05; 95% CI 0.02-0.13), whereas *CETP* 279GA (76% versus 28%; *p*<0.0001; OR=8.36; 95% CI 4.108-17.00) and *CETP* 279AA (12% versus 1%; *p*=0.003; OR=13.20; 95% CI 1.61-108.17) genotypes were more frequent between SCA subjects (Table 11 and Figure 19.B).

Allele frequencies of *CETP* 451G (wt) polymorphism were significantly higher in controls than in SCA subjects (74% versus 98%; *p*<0.0001; OR=0.07; 95% CI 0.03-0.19), in contrast allele frequencies of *CETP* 451A (mutant) variant was more frequent in SCA subjects (26% versus 2%; *p*<0.0001; OR=14.04; 95% CI 14.5.34-36.89) (Table 10 and Figure 19.C). *CETP* 451GG genotype was significantly higher in controls than among SCA subjects (63% versus 90%; *p*<0.0001; OR=0.19; 95% CI 0.08-0.43), while *CETP* 451AA mutant genotype was more frequent between SCA subjects (16% versus 0%; *p*<0.0001) (Table 11 and Figure 19.D).

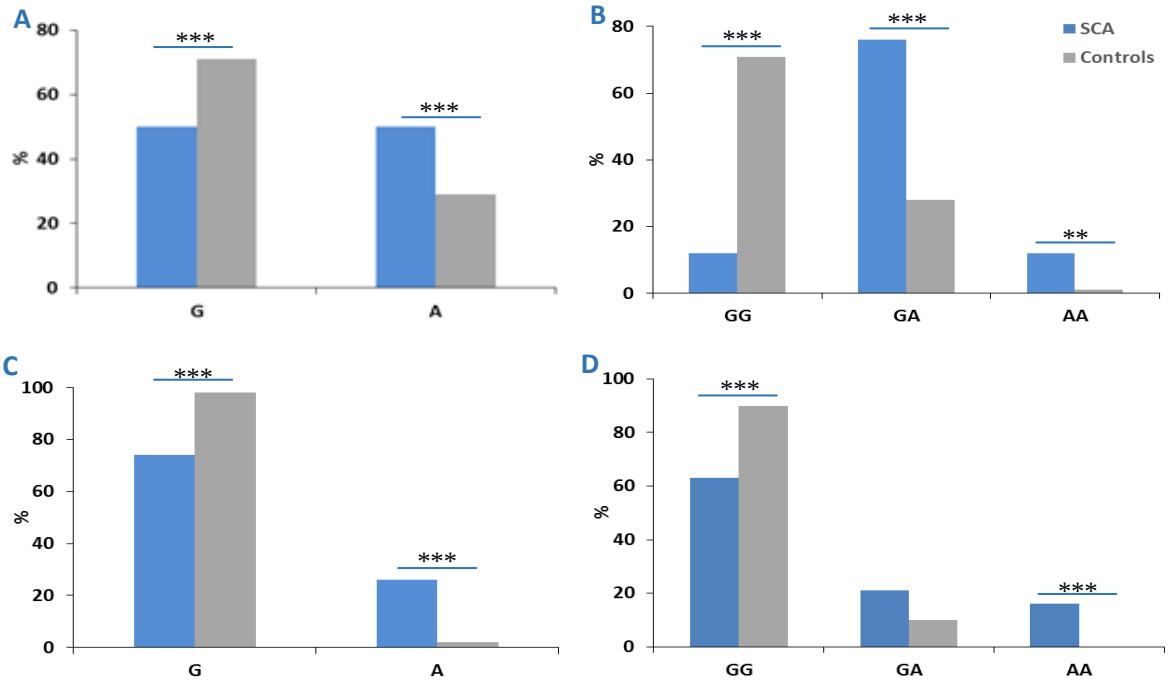

Figure 19. Allele and genotype frequencies of *CETP* gene polymorphisms among SCA and controls subjects: **(A)** Allele frequencies and **(B)** Genotype frequencies of *CETP* G279A; **(C)** Allele frequencies and **(D)** Genotype frequencies of *CETP* R451Q (G>A) (**p≤0.001; ***p≤0.0001).

Table 10. Allele frequencies distribution of *CETP* G279A and *CETP* R451Q (G>A) among SCA and control subjects.

Allele Frequencies	SCA and Controls						
	SCA		Controls		*p-value*	95 % CI	
	n	%	n	%		OR	RR
***CETP* G279A**							
G	68	50	142	71	**0.0001***	0.41 (0.26-0.64)	0.60 (0.47-0.77)
A	68	50	58	29		2.45 (1.55-3.86)	1.67 (1.29-2.15)
Total	136	100	200	100			
***CETP* R451Q**							
G	100	74	195	98	**<0.0001***	0.07 (0.03-0.19)	0.39 (0.32-0.47)
A	36	26	5	2		14.04 (5.34-36.89)	2.59 (2.13-3.15)
Total	136	100	200	100			

*Statistically significant

Table 11. Genotype frequencies distribution of *CETP* G279A and *CETP* R451Q (G>A) among SCA and control subjects.

Genotype Frequencies	SCA and Controls						
	SCA		Controls		*p*-value	95 % CI	
	n	%	n	%		OR	RR
***CETP* G279A**							
GG	8	12	71	71	**<0.0001***	0.05 (0.02-0.13)	0.15 (0.08-0.29)
GA	52	76	28	28		8.36 (4.11-17.00)	3.58 (2.23-5.73)
AA	8	12	1	1	**0.003***	13.20 (1.61-108.17)	2.36 (1.74-3.20)
Total	68	100	100	100			
***CETP* R451Q**							
GG	43	63	90	90	**<0.0001***	0.19 (0.08-0.43)	0.45 (0.33-0.63)
GA	14	21	10	10	n.s	2.33 (0.97-5.62)	1.56 (1.04-2.32)
AA	11	16	0	0	**<0.0001***	Infinity (NaN-Infinity)	2.75 (2.24-3.39)
Total	68	100	100	100			

*Statistically significant; n.s: not significant

3.3.3. *NPY* Leu7Pro, *PON1* Q192R (A>G) and *PPARG* Pro12Ala polymorphisms distribution among SCA and controls

Allele frequencies of *NPY* 7T (wt) polymorphism were higher in controls than in SCA subjects (73% versus 98%; $p<0.0001$; OR=0.05; 95% CI 0.02-0.16), while allele frequencies *NPY* 7C (mutant) polymorphism was more frequent between SCA subjects (27% versus 2%, $p<0.0001$; OR=18.31; 95% CI 6.35-52.83) (Table 12 and Figure 20.A). *NPY* 7TT wt genotype was significantly higher in controls than among SCA subjects (60% versus 96%; $p<0.0001$; OR=0.06; 95% CI 0.02-0.19), however *NPY* 7TC (25% versus 4%; $p<0.0001$; OR=8; 95% CI 2.56-25.04) and *NPY* 7CC (15% versus 0%; $p<0.0001$) genotypes were more frequent in SCA subjects (Table 13 and Figure 20.B).

Allele frequencies *PON1* 192A (wt) polymorphism was higher in controls than among SCA subjects (62% versus 83%; $p<0.0001$; OR=0.33; 95% CI 0.20-0.55). On the other hand, allele frequencies *PON1* 192G (mutant) was more prevalent between SCA subjects (38% versus 17%, $p<0.0001$; OR=3.02; 95% CI 1.82-5.01) (Table 12 and Figure 20.C). *PON1* 192AA genotype was significantly higher in controls than in SCA subjects (34% versus 72%; $p<0.0001$; OR=1.00; 95% CI 0.10-0.39), while *PON1* 192AG genotype was more frequent between SCA subjects (56% versus 22%; $p<0.0001$; OR=4.49; 95% CI 2.29-8.80) (Table 13 and Figure 20.D).

Finally, allele frequencies of *PPARG* 12C (wt) polymorphism are higher in controls than in SCA subjects (85% versus 93%; $p<0.0001$; OR=0.41; 95% CI 0.20-0.84), while allele frequencies of *PPARG* 12G (mutant) polymorphism was more prevalent between SCA subjects (15% versus 7%, $p<0.016$; OR=2.426; 95% CI 1.187-4.960) (Table 12 and Figure 20.E). *PPARG* 12GG genotype was more frequent among SCA subjects (10% versus 0%; $p=0.001$) (Table 13 and Figure 20.F).

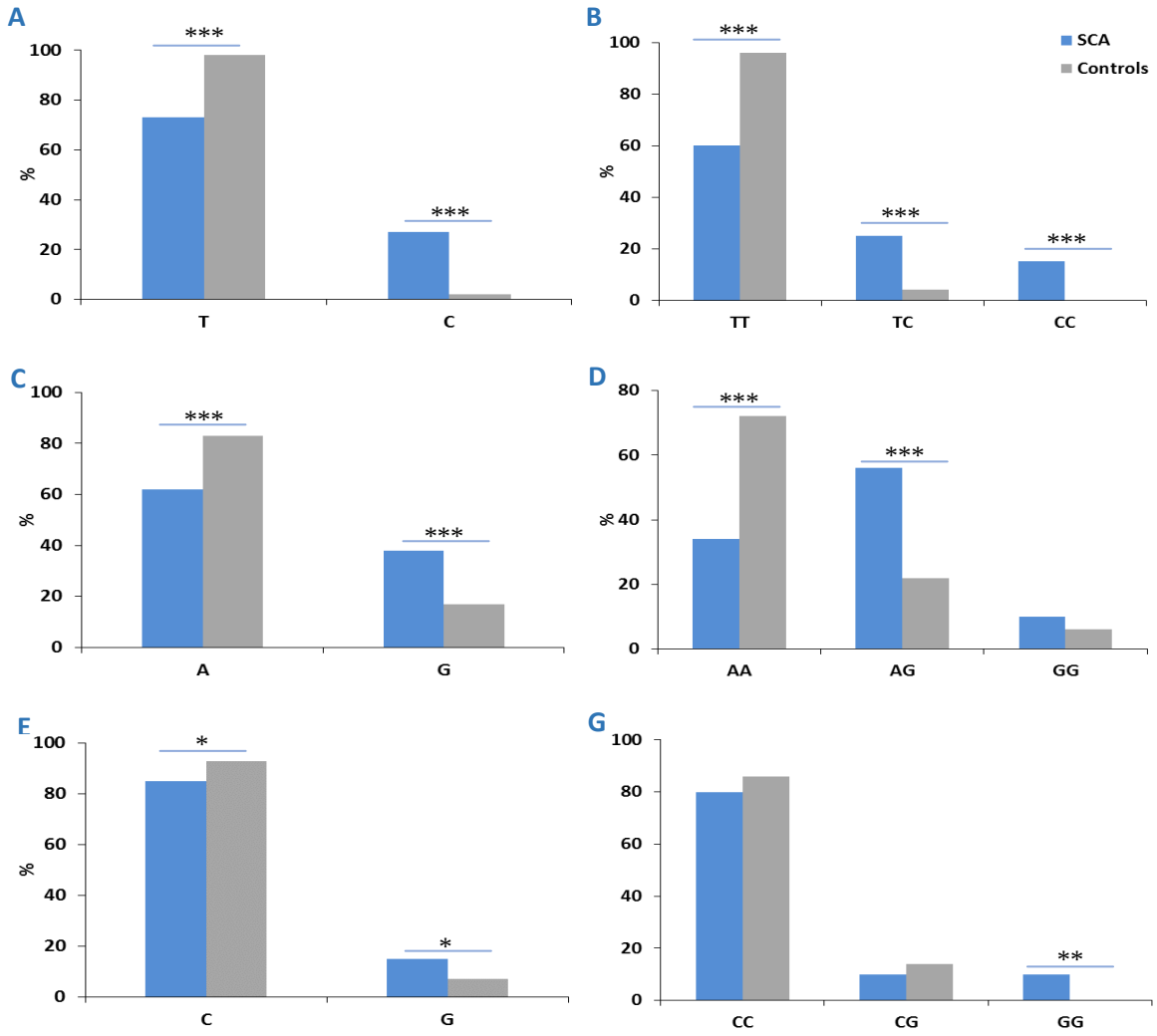

Figure 20. Allele and genotype frequencies distribution among SCA and controls subjects: **(A)** Allele and **(B)** Genotype frequencies of *NPY* Leu7Pro polymorphism; **(C)** Allele and **(D)** Genotype frequencies of *PON1* Q192R (A>G) polymorphism; **(E)** Allele and **(F)** Genotype frequencies of *PPARG* Pro12Ala polymorphism (*p≤0.05; **p≤0.001; ***p≤0.0001).

Table 12. Allele frequencies distribution of *NPY* Leu7Pro, *PON1* Q192R (A>G) and *PPARG* Pro12Ala among SCA and control subjects.

Allele Frequencies	SCA and Controls						
	SCA		Controls		*p-value*	95 % CI	
	n	%	n	%		OR	RR
***NPY* Leu7Pro**							
T	99	73	196	98	**<0.0001***	0.05 (0.02-0.16)	0.37 (0.31-0.45)
C	37	27	4	2		18.31 (6.35-52.83)	2.69 (2.22-3.25)
Total	136	100	200	100			
***PON1* Q192R**							
A	84	62	166	83	**<0.0001***	0.33 (0.20-0.55)	0.56 (0.44-0.71)
G	52	38	34	17		3.02 (1.82-5.01)	1.80 (1.41-2.30)
Total	136	100	200	100			
***PPARG* Pro12Ala**							
C	115	85	186	93	**0.02***	0.41 (0.20-0.84)	0.64 (0.47-0.87)
G	21	15	14	7		2.43 (1.19-4.96)	1.57 (1.16-2.13)
Total	136	100	200	100			

*Statistically significant

Table 13. Genotype frequencies distribution of *NPY* Leu7Pro, *PON1* Q192R (A>G) and *PPARG* Pro12Ala among SCA and control subjects.

Genotype Frequencies	SCA and Controls						
	SCA		Controls		*p*-value	95 % CI	
	n	%	n	%		OR	RR
***NPY* Leu7Pro**							
TT	41	60	96	96		0.06 (0.02-0.19)	0.34 (0.26-0.46)
TC	17	25	4	4	**<0.0001***	8.00 (2.56-25.04)	2.33 (1.72-3.16)
CC	10	15	0	0		Infinity (NaN-Infinity)	0.37 (0.30-0.45)
Total	68	100	100	100			
***PON1* Q192R**							
AA	23	34	72	72	**<0.0001***	0.20 (0.10-0.39)	0.39 (0.26-0.59)
AG	38	56	22	22		4.49 (2.29-8.80)	2.28 (1.59-3.27)
GG	7	10	6	6	n.s	1.80 (0.58-5.60)	1.37 (0.80-2.35)
Total	68	100	100	100			
***PPARG* Pro12Ala**							
CC	54	80	86	86	n.s	0.63 (0.28-1.42)	0.77 (0.50-1.18)
CG	7	10	14	14		0.70 (0.27-1.85)	0.80 (0.43-1.56)
GG	7	10	0	0	**0.001***	Infinity (NaN-Infinity)	2.64 (2.17-3.22)
Total	68	100	100	100			

*Statistically significant; n.s: not significant

3.4. Lipid metabolism genes polymorphisms in SCA stratified by clinical pathological features

3.4.1. Gender

Polymorphisms distribution of lipid metabolism in SCA subjects between males and female (genders) did not show statistically significant differences among them (Table 14 and 15).

Table 14. Allele frequencies distribution between genders.

Allele Frequencies	Gender						
	Male		Female		*p-value*	95 % CI	
	n	%	n	%		OR	RR
***APOA1* -75 G>A**							
G	56	55	15	44	n.s	0.65 (0.30-1.42)	0.72 (0.40-1.30)
A	46	45	19	56		1.54 (0.71-3.37)	1.38 (0.77-2.49)
Total	102	100	34	100			
***APOB* R3500Q**							
G	79	77	29	85	n.s	1.69 (0.59-4.86)	1.50 (0.64-3.53)
A	23	23	5	15		0.59 (0.21-1.70)	0.67 (0.28-1.56)
Total	102	100	34	100			
***APOC3* C3175G**							
C	53	52	21	62	n.s	1.49 (0.68-3.30)	1.35 (0.74-2.48)
G	49	48	13	38		0.67 (0.30-1.48)	0.74 (0.40-1.35)
Total	102	100	34	100			
***APOC3* T3206G**							
T	75	74	22	65	n.s	0.66 (0.29-1.51)	0.74 (0.41-1.34)
G	27	26	12	35		1.52 (0.66-3.47)	1.36 (0.75-2.47)
Total	102	100	34	100			
***APOE* Cys112/158Arg**							
E2	10	10	3	9	n.s	0.89 (0.23-3.44)	0.92 (0.32-2.59)
E3	80	78	26	76		0.89 (0.36-2.25)	0.92(0.47-1.82)
E4	12	12	5	15		1.29 (0.42-3.98)	1.22 (0.54-2.69)
Total	102	100	34	100			
***CETP* G279A**							
G	54	63	14	41	n.s	0.62 (0.28-1.37)	0.70 (0.39-1.27)
A	48	47	20	59		1.61 (0.73-3.53)	1.43 (0.79-2.59)
Total	102	100	34	100			

n.s: not significant

Table 14. Allele frequencies distribution between genders (cont).

***CETP* R451Q**							
G	73	72	27	79	n.s	1.53 (0.60-3.91)	1.39 (0.66-2.91)
A	29	28	7	21		0.65 (0.26-1.66)	0.72 (0.26-1.66)
Total	102	100	34	100			
***NPY* Leu7Pro**							
T	71	70	28	82	n.s	2.04 (0.77-5.42)	1.74 (0.79-3.87)
C	31	30	6	18		0.49 (0.18-1.30)	0.57 (0.26-2.02)
Total	102	100	34	100			
***PON1* Q192R**							
A	61	60	23	68	n.s	1.41 (0.62-3.19)	1.29 (0.69-2.43)
G	41	40	11	32		0.71 (0.31-1.62)	0.77 (0.41-1.45)
Total	102	100	34	100			
***PPARG* Pro12Ala**							
C	88	86	27	79	n.s	0.61 (0.22-1.68)	0.70 (0.35-1.40)
G	14	14	7	21		1.63 (0.60-4.45)	1.42 (0.71-2.83)
Total	102	100	34	100			

n.s: not significant

Table 15. Genotype frequencies distribution between genders.

Genotype Frequencies	**Gender**						
	Male		**Female**		***p-value***	**95 % CI**	
	n	**%**	**n**	**%**		**OR**	**RR**
***APOA1* -75 G>A**							
GG	20	39	5	29		0.65 (0.20-2.11)	0.72 (0.29-1.80)
GA	16	31	5	29	n.s	0.91 (0.27-3.02)	0.93 (0.38-2.31)
AA	15	30	7	42		1.68 (0.54-5.2)	1.46 (0.64-3.33)
Total	51	100	17	100			
***APOB* R3500Q**							
GG	32	63	13	76		1.93 (0.55-6.78)	1.66 (0.61-4.52)
GA	15	29	3	18	n.s	0.51 (0.13-2.05)	0.60 (0.19-1.83)
AA	4	8	1	6		0.73 (0.08-7.06)	0.79 (0.13-4.78)
Total	51	100	17	100			
***APOC3* C3175G**							
CC	13	25	6	35		1.59 (0.49-5.18)	1.41 (0.61-3.26)
CG	27	53	9	53	n.s	1.00 (0.33-3.00)	1.00 (0.44-2.28)
GG	11	22	2	12		0.48 (0.10-2.45)	0.56 (0.15-2.17)
Total	51	100	17	100			

n.s: not significant

Table 15. Genotype frequencies distribution between genders (cont).

***APOC3* T3206G**							
TT	32	63	10	59		0.85 (0.28-2.60)	0.88 (0.38-2.03)
TG	11	21	2	12	n.s	0.48 (0.10-2.45)	0.56 (0.15-2.17)
GG	8	16	5	29		2.24 (0.62-8.12)	1.76 (0.75-4.13)
Total (68)	51	100	17	100			
***APOE* Cys112/158Arg**							
E2/E2	2	4	1	6		1.53 (0.13-18.03)	1.35 (0.26-7.09)
E2/E3	5	10	0	0		0 (0-NaN)	0 (0-NaN)
E2/E4	1	2	1	6	n.s	3.13 (0.18-52.87)	2.06 (0.48-8.79)
E3/E3	32	63	11	65		1.09 (0.35-3.42)	1.07 (0.45-2.53)
E3/E4	11	21	4	23		1.12 (0.30-4.12)	1.09 (0.415-2.848)
E4/E4	0	0	0	0		0	0
Total	51	100	17	100			
***CE<TP* G279A**							
GG	7	14	1	6		0.39 (0.04-3.45)	0.47 (0.07-3.07)
GA	40	78	12	71	n.s	0.66 (0.19-2.28)	0.74 (0.31-1.78)
AA	4	8	4	23		3.62 (0.79-16.46)	2.31 (0.99-5.36)
Total	51	100	17	100			
***CETP* R451Q**							
GG	31	61	12	70		1.55 (0.47-5.06)	1.40 (0.56-3.50)
GA	11	21	3	18	n.s	0.78 (0.19-3.21)	0.83 (0.28-2.48)
AA	9	18	2	12		0.62 (0.12-3.21)	0.69 (0.18-2.60)
Total	51	100	17	100			
***NPY* Leu7Pro**							
TT	29	57	12	70		1.82 (0.56-5.93)	1.58 (0.63-3.98)
TC	13	25	4	24	n.s	0.90 (0.25-3.25)	0.92 (0.35-2.45)
CC	9	18	1	6		0.29 (0.03-4.49)	0.36 (0.05-2.44)
Total	51	100	17	100			
***PON1* Q192R**							
AA	16	31	7	41	n.s	1.53 (0.49-4.75)	1.37 (0.60-3.13)
AG	29	57	9	53		0.85 (0.28-2.57)	0.89 (0.39-2.02)
GG	6	12	1	6		0.47 (0.05-4.20)	0.55 (0.09-3.51)
Total	51	100	17	100			
***PPARG* Pro12Ala**							
CC	41	80	13	76		0.79 (0.21-2.96)	0.84 (0.32-2.19)
CG	6	12	1	6	n.s	0.47 (0.05-4.20)	0.54 (0.08-3.51)
GG	4	8	3	18		2.52 (0.50-12.62)	1.87 (0.71-4.93)
Total	51	100	17	100			

n.s: not significant

3.4.2. Tumour localization

Allele frequencies from *APOA1, APOB, APOC3, APOE, CETP, NPY, PON1* and *PPARG* common polymorphisms in colon SCA samples and rectum SCA samples are presented in Table 16. The majority those genes did not present a significant difference between groups, only *PPARG* had registered significant difference (Table 16).

Table 16. Allele frequencies distribution in colon versus rectum SCA.

Allele Frequencies	Tumour Localization						
	Colon		Rectum		*p-value*	95 % CI	
	n	%	n	%		OR	RR
***APOA1* -75 G>A**							
G	31	50	40	54	n.s	1.18 (0.60-2.31)	1.08 (0.79-1.47)
A	31	50	34	46		0.85 (0.43-1.67)	0.93 (0.68-1.27)
Total	62	100	74	100			
***APOB* R3500Q**							
G	52	84	56	76	n.s	0.60 (0.25-1.41)	0.81 (0.58-1.23)
A	10	16	18	24		1.67 (0.71-3.95)	1.24 (0.89-1.73)
Total	62	100	74	100			
***APOC3* C3175G**							
C	34	55	40	54	n.s	0.97 (0.49-1.91)	0.99 (0.72-1.34)
G	28	45	34	46		1.03 (0.52-2.03)	1.01 (0.75-1.38)
Total	62	100	74	100			
***APOC3* T3206G**							
T	46	74	51	69	n.s	0.77 (0.36-1.64)	0.89 (0.65-1.23)
G	16	26	23	31		1.30 (0.61-2.75)	1.12 (0.81-1.55)
Total	62	100	74	100			
***APOE* Cys112/158Arg**							
E2	7	11	6	8	n.s	0.69 (0.22-2.18)	0.83 (0.45-1.53)
E3	48	78	58	78		1.06 (0.47-2.38)	1.03 (0.70-1.50)
E4	7	11	10	14		1.23 (0.44-3.44)	1.09 (0.71-1.68)
Total	62	100	74	100			
***CETP* G279A**							
G	35	56	33	45	n.s	0.62 (0.315-1.225)	0.80 (0.59-1.10)
A	27	44	41	55		1.61 (0.82-3.18)	1.24 (0.91-1.70)
Total	62	100	74	100			
***CETP* R451Q**							
G	45	73	55	74	n.s	1.09 (0.51-2.35)	1.04 (0.73-1.49)
A	17	27	19	26		0.91 (0.43-1.96)	0.96 (0.67-1.37)
Total	62	100	74	100			

*Statistically significant; n.s: not significant

Table 16. Allele frequencies distribution in colon versus rectum SCA (cont).

***NPY* Leu7Pro**							
T	45	73	54	73	n.s	1.02 (0.48-2.18)	1.01 (0.71-1.43)
C	17	27	20	27		0.98 (0.46-2.09)	0.99 (0.70-1.40)
Total	62	100	74	100			
***PON1* Q192R**							
A	40	65	44	59	n.s	0.81 (0.40-1.62)	0.91 (0.67-1.24)
G	22	35	30	41		1.24 (0.62-2.49)	1.10 (0.81-1.50)
Total	62	100	74	100			
***PPARG* Pro12Ala**							
C	57	92	58	78	**0.04***	0.32 (0.11-0.93)	0.66 (0.49-0.89)
G	5	8	16	22		3.14 (1.08-9.16)	1.51 (1.12-2.04)
Total	62	100	74	100			

*Statistically significant; n.s: not significant

Allele frequencies *PPARG* 12C (wt) polymorphism was higher in colon SCA than in rectum SCA subjects (92% versus 78%; *p*=0.04; OR=0.32; 95% CI 0.11-0.93), while allele frequencies of *PPARG* 12G (mutant) polymorphism were more prevalent in rectum SCA subjects (8% versus 22%, *p*=0.04; OR=3.145; 95% CI 1.080-9.156) (Table 16 and Figure 21.A)

Allele frequencies from *PPARG* gene polymorphism in colon SCA versus controls subjects, and rectum SCA versus controls subjects were present in Table 17 and Table 18, respectively. Thus, allele frequencies *PPARG* polymorphism showed statistically significant differences between both rectum SCA and controls subjects groups and no difference between colon SCA and controls subjects. Allele frequencies *PPARG* 12C polymorphism were higher in controls than in rectum SCA samples (78% versus 93%; *p*=0.001; OR=0.273; 95% CI 0.126-0.593) (Table 18 and Figure 21.B). On other hand, *PPARG* 12G allele frequencies were more prevalent in rectum SCA subjects (22% versus 7%; *p*=0.001; OR=3.665; 95% CI 1.688-7.959).

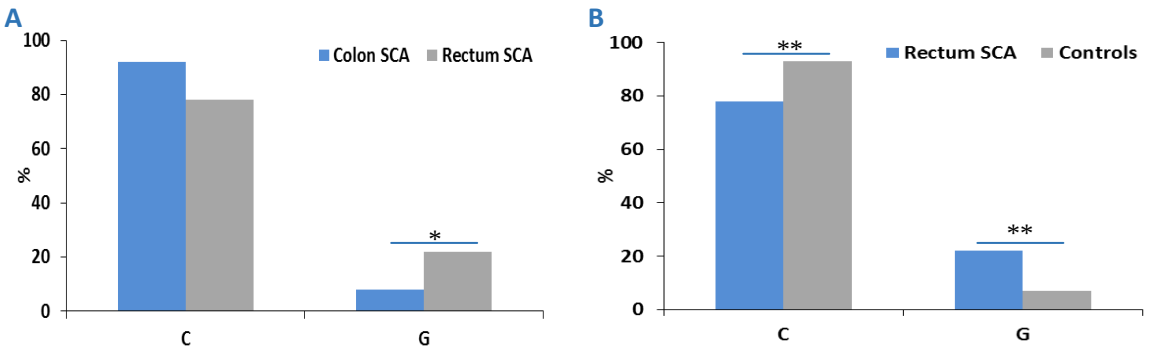

Figure 21. Allele frequencies of *PPARG* Pro12ala in **(A)** Colon SCA and Rectum SCA **(B)** Rectum SCA and control subjects (*p≤0.05; **p≤0.001).

Table 17. Allele frequencies of *PPARG* Pro12Ala distribution among colon SCA and controls subjects.

Allele Frequencies	Colon SCA and Controls						
	Colon SCA		Controls		*p-value*	95 % IC	
	n	%	n	%		OR	RR
***PPARG* Pro12Ala**							
C	57	92	186	93	n.s	1.17 (0.40-3.38)	1.04 (0.79-1.37)
G	5	8	14	7		0.86 (0.30-2.49)	0.96 (0.73-1.27)
Total	62	100	200	100			

n.s: not significant

Table 18. Allele frequencies of *PPARG* Pro12Ala distribution among rectum SCA and controls subjects.

Allele Frequencies	Rectum SCA and Controls						
	Rectum SCA		Controls		*p-value*	95 % IC	
	n	%	n	%		OR	RR
***PPARG* Pro12Ala**							
C	58	78	186	93	**0,001***	3.67 (1.69-7.96)	1.63 (1.11-2.41)
G	16	22	14	7		0.27 (0.13-0.59)	0.61 (0.41-0.90)
Total	74	100	200	100			

*Statistically significant

Genotype frequencies from *APOA1, APOB, APOC3, APOE, CETP, NPY, PON1* and *PPARG* common polymorphisms in colon SCA and rectum SCA samples are presented in Table 19. The majority of those genes did not presented a significant difference between groups, except *APOB* 3500GA, *APOC3* 3206TG, *CETP* 279AA genotypes had reported significant difference among groups (Table 19).

APOB 3500GA genotype was significantly higher in rectum SCA than in colon SCA samples (13% versus 38%; $p<0.03$; OR=4.11; 95% CI 1.19-14.235) (Table 19 and Figure 22.A). Also, *APOC3* 3206TG genotype was significantly higher in colon SCA than rectum SCA samples (32% versus 8%; $p<0.02$; OR=0.185; 95% CI 0.05-0.75) (Table 19 and Figure 22.B). In contrast, *CETP* 279AA genotype was only present in rectum SCA samples (0% versus 22%; $p<0.006$) (Table 19 and Figure 22.C).

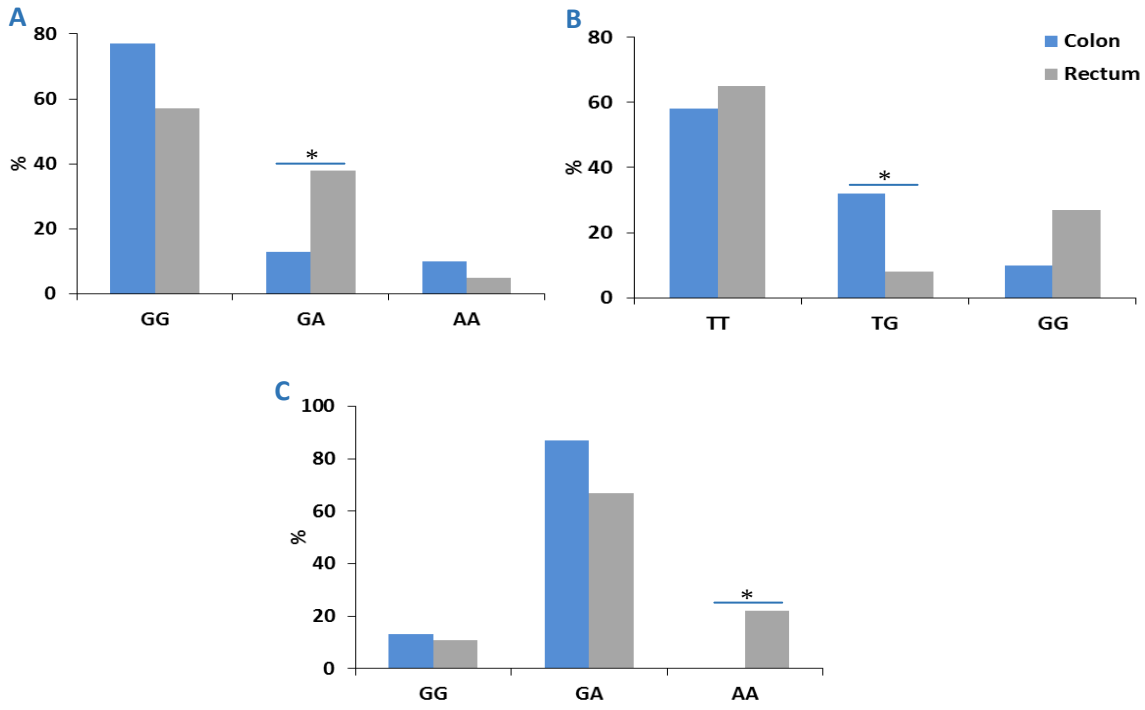

Figure 22. Genotype frequencies in Colon SCA and Rectum SCA to **(A)** *APOB* R3500Q, **(B)** *APOC3* T3206G and **(C)** *CETP* G279A (*p≤0.05).

Table 19. Genotype frequencies distribution in colon and rectum SCA samples.

Genotype Frequencies	Tumour Localization						
	Colon		Rectal		*p-value*	95 % CI	
	n	%	n	%		OR	RR
***APOA1* -75 G>A**							
GG	9	29	16	43		1.86 (0.68-5.12)	1.31 (0.86-2.00)
GA	13	42	8	22	n.s	0.38 (0.13-1.10)	0.62 (0.34-1.11)
AA	9	29	13	35		1.32 (0.47-3.70)	1.13 (0.73-1.77)
Total	31	100	37	100			
***APOB* R3500Q**							
GG	24	77	21	57	n.s	0.38 (0.13-1.11)	0.67 (0.44-1.01)
GA	4	13	14	38	**0.03***	4.11 (1.19-14.24)	1.69 (1.15-2.49)
AA	3	10	2	5	n.s	0.53 (0.08-3.42)	0.72 (0.24-2.15)
Total	31	100	37	100			
***APOC3* C3175G**							
CC	9	29	10	27		0.91 (0.31-2.62)	0.96 (0.58-1.57)
CG	16	52	20	54	n.s	1.10 (0.42-2.87)	1.05 (0.68-1.62)
GG	6	19	7	19		0.97 (0.29-3.27)	0.99 (0.56-1.73)
Total	31	100	37	100			
***APOC3* T3206G**							
TT	18	58	24	65	n.s	1.33 (0.50-3.56)	1.14 (0.72-1.82)
TG	10	32	3	8	**0.02***	0.19 (0.05-0.75)	0.37 (0.14-1.03)
GG	3	10	10	27	n.s	3.46 (0.86-13.94)	1.57 (1.05-2.34)
Total	31	100	37	100			
***APOE* Cys112/158Arg**							
E2/E2	2	6	1	3		0.40 (0.03-4.67)	0.60 (0.12-3.03)
E2/E3	2	6	3	8		1.28 (0.20-8.19)	1.11 (0.52-2.36)
E2/E4	1	3	1	3	n.s	0.83 (0.05-13.90)	0.92 (0.23-3.73)
E3/E3	20	65	23	62		0.90 (0.34-2.44)	0.96 (0.61-1.49)
E3/E4	6	20	9	24		1.34 (0.42-4.30)	1.14 (0.70-1.85)
E4/E4	0	0	0	0		0	0
Total	31	100	37	100			
***CETP* G279A**							
GG	4	13	4	11	n.s	0.82 (0.19-3.58)	0.91 (0.44-1.89)
GA	27	87	25	67		0.31 (0.09-1.08)	0.64(0.43-0.96)
AA	0	0	8	22	**0.006***	Infinity (NaN-Infinity)	2.07 (1.59-2.69)
Total	31	100	37	100			
***CETP* R451Q**							
GG	19	61	24	65		1.17 (0.43-3.13)	1.07 (0.68-1.70)
GA	7	23	7	19	n.s	0.80 (0.25-2.60)	0.90 (0.51-1.60)
AA	5	16	6	16		1.01 (0.28-3.68)	1.00 (0.56-1.81)
Total	31	100	37	100			

Table 19. Genotype frequencies distribution in colon and rectum SCA samples (cont).

***NPY* Leu7Pro**							
TT	19	61	22	59		0.93 (0.35-2.46)	0.97 (0.62-1.50)
TC	7	23	10	27	n.s	1.27 (0.42-3.86)	1.11 (0.69-1.79)
CC	5	16	5	14		0.81 (0.21-3.11)	0.91 (0.47-1.76)
Total	31	100	37	100			
***PON1* Q192R**							
AA	11	36	12	32	n.s	0.87 (0.32-2.39)	0.94 (0.59-1.50)
AG	18	58	20	54		0.85 (0.32-2.23)	0.93 (0.60-1.43)
GG	2	6	5	14		2.27 (0.41-12.59)	1.36 (0.80-2.30)
Total	31	100	37	100			
***PPARG* Pro12Ala**							
CC	28	90	26	70		0.25 (0.06-1.01)	0.61 (0.42-0.90)
CG	1	3	6	16	n.s	5.81 (0.66-51.15)	1.69 (1.14-2.49)
GG	2	7	5	14		2.27 (0.41-12.59)	1.36 (0.80-2.30)
Total	31	100	37	100			

*Statistically significant; n.s: not significant

Chapter IV
Discussion

4. Discussion

CRC pathogenesis is a multistep process involving genetic and environmental factors that influence disease progression.[19] Between these factors, dietary[44,25] and genetic polymorphisms[8] are fundamental for SCA susceptibility. Furthermore, there are recent evidences that several SNP associated to SCA development may be influenced by high fat diet, lifestyle and other social habits.[133] Moreover, association studies have showed a relation between the presence of *APOA1 -75 G>A*[72], *APOB* R3500Q[84], *APOC3* C3175G[89], *APOC3* T3206G[57], *APOE* Cys112/158Arg[58], *CETP* G279A[60], *CETP* R451Q[59], *NPY* Leu7Pro[134], *PON1* Q192R[62], *PPARG* Pro12Ala[135] polymorphisms and the decrease of cholesterol and/or triglycerides plasma concentrations, as well as hyperlipidemia's.[136,137,138,139] However, there is not a previous approach to relate these genetic variants with CRC risk, except for *APOE*[93], *PPARG*[140] and *PON1*[62] polymorphisms.

Most tissues are not capable to catabolize cholesterol, when this occur the non-catabolized cholesterol returns into the liver. In liver cholesterol can be then secreted into bile, directly or after conversion to bile acids (BAs). This pathway might stimulate BAs secretion into the colon. BAs are cholesterol metabolites and are classified in two groups: primary BAs (BAs I) that are synthesized in liver bias cholesterol; and secondary BAs (BAs II) that are produced by intestinal bacteria bias Bas I. In colonic cells, BAs promotes digestion assuring fat solubilization and emulsification (Figure 24). After meal, BAs and their conjugates are delivered in duodenum to facilitate fats and vitamins digestion and absorption by the intestine throughout enterocyte barrier. In ileum and colon, the majority of BAs (95%) is reabsorbed into liver and then recycled (they can be excreted 20-40 times during digestion). Nevertheless, the excess of BAs can be eliminated by the feces, namely BAs II.[130,131,141,142,143]

Colon cells exposure to high physiologic BAs levels induces reactive oxygen species (ROS) formation and, consequently, cells undergo oxidative stress that could lead to DNA damage and chromosome abnormalities (Figure 23).[144,128] As result of DNA damage, apoptosis is inducted by p53 signaling pathway activation. P53 is a crucial tumour suppressor involved in several cellular responses, such as differentiation, senescence, DNA repair and angiogenesis inhibition.[145] However, a prolonged exposure to high BAs

levels of normal cells and the replication of cells with DNA damage originate mutant cells clusters. [128,136,146]

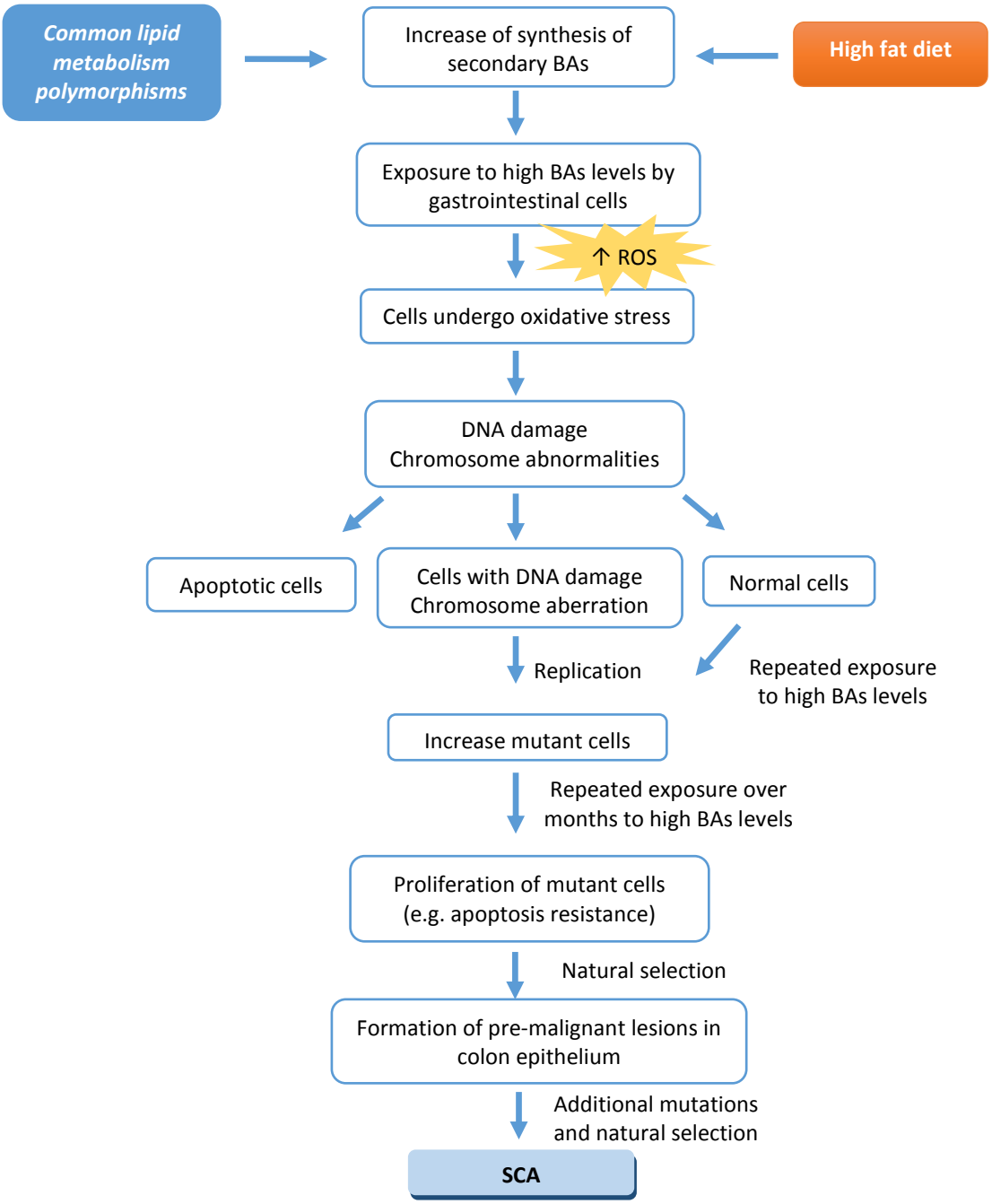

Figure 23. Diagram of SCA progression and development from increase of bile acid levels caused by genetic factors (SNP's) and high fat diet. Adapted from references[144,128]

In long-term, persistent colonic epithelial cells exposure to high BAs physiologic concentration appears to favor survival of the cells that are resistant to BAs apoptosis induction (resistance to p53 signaling pathway), leading to their proliferation (Figure 23). BAs II have influence in most genes involved in inflammation processes, angiogenesis, epithelial barrier function, oxidative stress, apoptosis, cell proliferation/cell cycle/DNA repair, membrane transport and ubiquitin-proteasome pathway. These mechanisms lead to secondary mutations formation that are involved in colorectal adenomas development.[128,146,129, 144,145]

APOA1 -75 G>A, *APOB* R3500Q, *APOC3* C3175G, *APOC3* T3206G, *APOE* Cys112/158Arg, *CETP* G279A, *CETP* R451Q, *PON1* Q192R, *NPY* Leu7Pro, *PPARG* Pro12Ala polymorphisms have not their role in BAs II cancer mechanisms well clarified although there are some evidences that associate these variants with cholesterol and/or triglycerides metabolism that are directly involved in BAs metabolic regulation. [55,56,57,58,59,60,61,62,63] In this work we tried to make a connection between lipid metabolism gene polymorphisms, diet cholesterol/BAs equilibrium, adenomas development and SCA risk (Figure 23).

4.1. Influence of common lipid metabolism genes polymorphism among SCA and controls

Lipid transport and metabolism genes studied are involved in essentials mechanisms of SCA development and progression. This hypothesis is corroborated with the results of this work, since it was found a high correlation between mutant alleles, *APOA1* -75A, *APOB* 3500Q, *CETP* 279A, *CETP* 451Q, *APOC3* 3175G, *PON1* 192R, *NPY* 7Pro and *PPARG* 12Ala, and SCA prevalence. However, *APOE* gene and *APOC3* T3206G variant only demonstrated a significate differences in their genotypes. All these genes are linked to dyslipidemia. Dyslipidemias are usually associated with intestinal mucosal exposure to high lipids concentrations, including cholesterol and BAs II.[139,9]

4.1.1. Influence of APOA1 polymorphism in SCA

APOA1 is the major HDL-C protein component and plays a crucial role in reverse cholesterol transport, promoting cholesterol efflux from tissues into liver. Also, it is a cofactor for LCAT which is responsible for most plasma cholesteryl esters formation.[68,47] Defects in *APOA1* gene are associated with HDL alterations, bias influence of apolipoprotein expression levels. *APOA1* -75A allele is linked to increase *APOA1* gene transcription levels, thus individuals carrying this allele have higher APOA1 and HDL-C concentrations. However, this relationship with APOA1 and HDL-C levels is controversial as indicated by inconsistencies in well-designed studies. *Danek et al,*[65] suggest that *APOA1* -75A allele does not directly affect *APOA1* gene transcriptional efficiency.[65] Other studies have shown that *APOA1* -75AA genotype was associated with type 2 diabetes increased risk, on the contrary *APOA1* -75GG genotype has been found to be strongly associated with myocardial infarction, hypertension and metabolic syndromes. This finding suggest that APOA1 -75A allele carriers have a lower lipid absorption in intestinal mucosa.[55] Several studies revealed essential APOA1 roles in inflammation, tumour growth, angiogenesis, invasion and metastasis. The increased levels of APOA1 in patients with adenocarcinomas were associated with enhanced tumour growth and lymphatic invasion. *Hamrita et al*,[69] show that *APOA1* -75 G>A have an important role in susceptibility to breast cancer metastases occurrence.[69] Nevertheless, there is no association yet between *APOA1* -75 G>A variant and CRC risk, since this polymorphism has been only associated with cardiovascular events .[65]

Although most studies are contradictory, in this work we found that *APOA1* -75A allele and *APOA1* -75AA genotype carriers have a strong association with SCA prevalence. *APOA1* -75A mutation appears to be associated with lipids low absorption by enterocytes and with increased fats accumulation in colon and rectum. Also, *APOA1* -75A is correlated with higher HDL and low LDL blood levels[7], and since HDL transport to hepatocytes has showed an increase bile acid synthesis,[147] its association with an increased risk of SCA is justified. Moreover, there are also evidences that this variant could manipulate BAs II and inflammation levels,[69] mechanisms correlated with p53 inhibition and SCA risk increase.

4.1.2. Influence of APOB polymorphism in SCA

APOB is the main apolipoprotein of chylomicrons, VLDL, IDL, and LDL particles, which allows lipid transport to all human tissues. APOB-100 isoform is express in liver and is an essential component for VLDL synthesis and secretion, also can acts as a ligand for LDL recognition and catabolism by its receptor. [47,80] Mutation in APOB-100 cause changes in LDL and VLDL synthesis and transport. Several finding have shown that *APOB* R3500Q variant change protein functional activity, decreasing its binding LDL receptor, slowing LDL particles clearance and leading LDL-C blood accumulation. All *APOB* R3500Q (G>A) genotypes presented statistically significate differences among SCA and control subjects, but heterozygous (GA) and mutate homozygous (AA) were more frequent among SCA subjects. Thus, *APOB* 3500Q (A) allele appears to be a potential SCA risk factor. A possible explanation for these findings is the lipids accumulation in colon, including cholesterol and BAs II, which contributes to ROS and oxidative stress increase.

Our results are in accordance with a study conducted in vitro in rat hepatocytes suggesting that 3500A allele, although being associated to LDL-C increase in circulation, duo to its LDL-R low affinity, also decrease triglycerides and LDL-C transport to liver or colon for other tissues. Contrarily, wt homozygous, *APOB* 3500RR suggested has a protective effect in SCA.[148] Furthermore, *APOB 3500QQ* carriers normally are hypercholesterolemic, however TC, LDL-C and TG concentrations are lower in Familial defective apolipoprotein B-100 (FDB) than familial hypercholesterolemia (FH),[56] and lower plasma lipoprotein levels in FDB can partially be caused by an increased uptake of VLDL remants.[81] Also, APOB-100 secretion have effects on BAs synthesis, being this fact demonstrated in a study realized in HepG2 cells. The decrease of APOB-100 and the increase of APOA1 concentrations are normally accompanied by BAs synthesis increment.[147] Our work demonstrated that *APOB 3500Q* variant is associated SCA, since this mutation increases bile acid synthesis and its accumulation in colon and rectum disrupting p53 pathway.

4.1.3. Influence of APOC3 polymorphisms in SCA

APOC3 is a fundamental protein in triglycerides lipolysis inhibition, through LPL and hepatic TG lipase activity inhibition. This protein is a component of chylomicron and VLDL, that acts as a major triglycerides catabolism regulator, resulting in a diminished catabolic rate and in a reduced chylomicrons and VLDL clearance.[57,47,87,88] APOC3 activity is affected by two mutations in *APOC3* gene, C3175G and T3206G. In this study, heterozygous and mutant homozygotes of *APOC3* 3175 showed a strong association with SCA, which can be explained by their higher APOC3 activity when compared with wt genotype. Although this mutation has not been yet related with CRC, several studies demonstrate its association with cardiovascular diseases (CVD) and dyslipidemias. Numerous studies has investigated these polymorphism associations with APOC3 and lipid plasma levels. Accordingly, some studies demonstrated that the carriers *APOC3* 3175G mutant allele is associated with higher APOC3, TG, TC, and LDL-C plasma levels and lower HDL-C levels.[57,89] These low HDL-C plasma levels can explain the prevalence of this variant in SCA subjects, suggesting that it could be accumulating in colon.

In this work, *APOC3* T3206G variant seemed to influences SCA development. *APOC3* 3206GG mutant genotype showed a strong association with SCA risk increase, while *APOC3* 3206TG genotype showed a protective effect for SCA. *APOC3* 3206GG genotype has been associated with lower TC, HDL-C and LDL-C plasma levels, that could explain its prevalence among SCA subjects,[57,89] since these elements lower plasma levels are associated with higher lipid excretion by feces, resulting in lipids absorption decrease.[11,12,149] These two *APOC3* polymorphisms appear to have a similar effect, although by different pathways, on SCA susceptibility increasing its risk by increasing intestinal lipid accumulation and excretion through feces.

4.1.4. Influence of APOE polymorphisms in SCA

APOE plays an important role in lipid metabolism by mediating lipoprotein particles binding to LDL and APOE receptors. In this work, *APOE* E3/E4 genotype showed a negative association with SCA prevalence. This can be explain by APOE's role in entero-

hepatic metabolism, influencing fecal bile acids concentration, which may participate in CRC adenocarcinomas formation, essentially in proximal colon, where they are in direct contact with the mucous membrane. *APOE E4/E4* genotype seemed to be a protective factor in SCA, being only present in control subjects. *APOE*4* allele presences is associated with lower concentrations secondary bile acids in gastrointestinal tract.[41,58,93] It suggest that *APOE* E4 allele presence results in higher cholesterol absorption and lower intestinal accumulation.[41,58,93] *APOE* E4/E4 protective effect is confirm by several studies realized with CRC patients.[41,58,93] Nevertheless, *APOE E4* allele has been associated with increased risk in other diseases, including hypertension, coronary disease, breast cancer and Alzheimer.[90,91]

4.1.5. Influence of CETP polymorphisms in SCA

CETP facilitates cholesteryl esters transfer from HDL to APOB-containing lipoproteins in exchange of triglycerides.[51] Furthermore, CETP plays an important role in reverse cholesterol transport pathway. In this work, *CETP* 279AA, mutant variant, and *CETP* 279GA genotypes showed significant associations with SCA occurrence. A previous study, in children from families with cardiovascular system diseases history, showed a correlation between *CETP* 279A allele presence, CETP lower activity and higher HDL cholesterol value. Moreover, this study also showed that *CETP* 279GA and *CETP* 279AA are linked to TG, TC, LDL-C, VLDL-C and APOB levels decrease, on other hand they are associated with APOA1 increase.[100] However there are no studies that relate this variant with CRC, nevertheless cardioprotective effects are normally associated with a lipid absorption reduction in gut and consequently increase BAs II levels within intestinal mucosa.

CETP gene has other genetic variant linked to CRC risk, R451Q (G>A). A study conducted on population of Northern Finland showed that increase in this mutation frequency was linked to CETP activity increase and lower cholesterol plasma levels.[59] This speculation may explain our results, in which *CETP* 451AA (QQ, mutant genotype) was prevalent between SCA subjects. Like *CETP* G279A there are no studies that relate

CETP R451Q with CRC, nevertheless, lower cholesterol serum levels associated with a decrease in lipid absorption by colon epithelium could explained this positive association among SCA and *CETP* variants.

4.1.6. Influence of NPY polymorphism in SCA

NPY is a neural transmitter that regulates food intake and energy storage. Also, it may regulate serum free fatty acid (FFA) levels. *NPY* Leu7pro polymorphism has been associated with serum TC and LDL-C regulation. *NPY* 7Pro allele carriers have lower FFA levels and higher lipid oxidation rates than *NPY* 7Leu allele carriers, however heterozygotes seemed to have a lower lipid oxidation rates.[52] This evidences could explain our results, since every genotypes exhibited statistically significate differences, being *NPY* 7ProLeu o and 7ProPro mutated genotypes associated with SCA risk, whereas 7LeuLeu (wt genotype) appears to have a protective role in SCA. Although NPY polymorphisms has not yet been studied in CRC, our results are in accordance with this variant role in NPY activity, since it mutated form increases NPY activity increasing food intake, dropping FFA levels and increasing lipid oxidation rates,[132,25] variables directly associated with CRC risk.

4.1.7. Influence of PON1 polymorphism in SCA

PON1 has a crucial role in HDL antioxidant activity by protecting LDL against lipid peroxidation. PON1 protein in plasma is co-associated with HDL, playing an atheroprotective effect. A study realized in mice demonstrate that increase bile acids alter PON1 hepatic expression, leading to PON1 repression and increasing inflammatory cytokines expression and secretion.[119] Genetic variations in antioxidant enzymes may influence colonic expression pattern and modulate colon epithelium cells ability to handle with metabolic damage and alterations. *PON1* Q192R (A>G) polymorphism is associated with altered PON1 concentrations. In this work, *PON1* 192A (Q) allele demonstrated to have a protective role in SCA and *PON1* 192G (R) allele and *PON1* 192AG (QR) genotype

showed an association with SCA risk. However, a study reported the opposite effect being *PON1* 192G (R) mutated allele associated with protection and the wt associated with risk.[62] Nevertheless, other study demonstrated that PON1 plasma concentration decrease drastically in CRC, comparatively with controls subjects, being is low activity associated with tumourgenesis.[118] PON1 in normal conditions have a protective role against oxidative damage and lipid peroxidation on colon cells.[115,150] Thus, the incidence of this polymorphism causes a higher susceptibility to lipid oxidation exposure and ROS accumulation in epithelium colon, increasing the likelihood of SCA prevalence.

4.1.8. Influence of PPARG polymorphism in SCA

PPARG regulate essential genes in cell differentiation, several metabolic processes, such as lipid and glucose homeostasis, and oxidative processes.[151] Moreover, it plays roles in lipid storage, inflammation and energy metabolism. *PPARG* gene exhibits different expression in normal colonic mucosa, colorectal adenocarcinomas and colon cancer cell lines. *PPARG* 12Ala (mutated form) polymorphism is related with PPARG decrease activity. In this work, *PPARG* 12Ala mutated allele and genotype showed an association with SCA risk. These results are in agreement with the literature, a study realized in CRC patients demonstrated that this mutation can have an protector effect in colon, but presents risk in rectum.[124] Thus, the incidence of this polymorphism causes a higher susceptibility to lipid oxidation and ROS accumulation increasing the SCA risk.

4.2. Influence of lipid transport and metabolism genes polymorphisms in SCA

Most of the polymorphisms approached in this work seemed to influence SCA apparently by lipids and triglycerides levels increase and/or oxidation rates raise. A disturbance in genes involved in lipid metabolism and transport, may cause less lipids absorption by colonic mucosa. This pathway also impairs enterohepatic cycle, which has an important role in bile acids metabolism, increasing BAs I production and consequently increases BAs II formation. Prolonged exposure of colonic epithelium to high BAs

concentrations rises: lipid peroxidation; ROS production; cell membrane rupture; DNA damage induced by mitochondria; and mutation levels.[128,144] This prolonged exposure also reduction cells apoptosis capacity normally induced by cellular genomic instability (Figure 23), increasing cell membrane permeability, leading to colonic epithelium destruction. Consequently, these elements induce cell proliferation by activating epidermal growth factor receptor (EGFR) and others growth factors.[146] Furthermore, BAs induced hyperproliferation can occur through the activity of protein kinase C (PKC), which can be activated by EGFR downstream or by membrane perturbation. ROS/RNS production has several consequences, such as: mitochondrial membrane alterations, high mutations occurrence levels and apoptosis intrinsic pathway activation (by tumour suppressor p53). [146] This pathway, leads to cytochrome C release and cytosolic caspases stimulation. Furthermore, BAs can also induce *K-ras* mutations, and micronuclei aneuploidy formation. All of these processes, induced by BAs, are described to influence positively tumourigenesis process, namely SCA.[128,129,145,146]

4.3. Gender

It appeared to be no link between any polymorphic variants and gender, since there was no significant differences in allele and genotype distributions among both genders. These results can be possibly explained by the fact that the majority of our samples are composed by male subjects (75% versus 25%).

4.4. Distinction between colonic and rectal adenocarcinomas

Colon cells are subject to a high cell renewal.[131] Colonic epithelium persistent exposure to elevated lipid concentrations and bile acids, together with genetic factors, leads to intestinal adenomas formation.[128,139] BAs effects varies with intestinal location, colon or rectum. This could be explained by BAs I and BAs II synthetizes location, in liver and colon, respectively.[131] Also, BAs II seem to have a greater carcinogenic promotion among rectum tissue. Therefore, significant differences between rectal and colon carcinomas with respect to genetic features have been reported.[152]

SCA subjects in this study were stratified according to primary tumour location: colonic or rectal. This work showed that *PPARG* 12Ala polymorphism was associated to rectum tumour. This result is in accordance with the majority of studies, although not significant, that reported that *PPARG* 12AlaAla is associated with increased rectum cancer risk.[63,124] In addition, also was reported that *PPARG* 12Ala variant is a protective factor in colon cancer. Results from this study showed that *APOC3* 3206TG genotype was associated with colon tumour. This result can be explained by expression level among colon and rectum, since *APOC3* gene has a major expression in colon normal tissue.[153]

Moreover, *APOB* 3500GA and *CETP* 279AA (mutant) genotypes shows a stronger association with rectum tissue. *Sniderman et al*.[147] demonstrate that decrease APOB levels was associated with bile acids formation that it has a major influence in rectum, suggesting an implication in CRC risk.[147] One study showed that CETP secretion is regulated by fatty acids absorption into Caco-2 cells.[154] *CETP* 279A variant is associated with CETP decreased activity, which is also decreased in patients with cholestasis (pathology associated with nutritional deficiencies due to inadequate dietary intake, maldigestion, malabsorption and/ or poor metabolism of nutrients).[154] This finding suggests that CETP have a similar mechanism in SCA and cholestasis, which proposes enterohepatic recycling impairment causing fats and bile acids accumulation in liver and intestine, increasing their excretion through rectum.[131,154] Again prolonged exposure to fats can increase ROS and consequential cells damaging. This work reinforces that there are some susceptibility differences according to tumour primary localization.

4.5. Future perspectives

In future could be fundamental gathered investigations about fat and BAs II levels in feces to certificate the association between SCA prevalence and increased lipid and ROS levels and excretion among gastrointestinal tract. Also is essential to get CRC patient's HDL-C, LDL-C, TG and TC plasma levels and relate their decrease with the increase of lipid levels in feces. Finally, is important to relate the polymorphisms studied with their gene and protein expressions, determining if occur loss or gain-of-function, and allowing the clarification of polymorphisms role in SCA.

4.6. Study limitations

There may be several possible mechanisms underlying all association studies, such as, the results from interaction of both environmental and genetic factors, which can be responsible for analysis default. Present study sample size (68 SCA and 100 controls) may not be large enough to detect small effect from low penetrance mutations. The combined effect of multiple genes/mutations can provide more reliable information for genetic contribution to SCA risk. We cannot completely exclude the effects from other conditions (i.e. weight, gender, diet type, etc.) and residual confounding attributable to the measurement error (namely, unicentric characters, takes medication, lack of assess of diet in takes, etc…). It is essential a large approach study with large sample size to confirm our outcomes. Still, the present study provides preliminary evidence that *APOA1, APOB, APOC3, CETP, NPY, PON1* and *PPARG* variants, may contribute to the SCA risk in Portuguese population, and may be useful tools in the study of this multifactorial disorder.

Chapter V
Conclusions

5. Conclusion

Observations and results founded in this study show that polymorphisms from *APOA1, APOB, APOC3, CETP, NPY, PON1* and *PPARG* genes were associated with increased risk development of SCA. This leads to believe that individuals carrying these genetic variants may differently susceptible to SCA. Common *APOE* gene polymorphism revelled has a protective effect in SCA. APOA1, APOB, APOC3, CETP, NPY, PON1 and PPARG proteins, whose role is essential in lipid transport and metabolism and also in antioxidant properties (PON1 and PPARG), when altered can influenced adenocarcinoma development process in colon and rectum tissues. Alterations in genes encoding proteins involved in lipid metabolism and transport contribute their functions deregulation. There are several mechanisms that may explain how this change may affect CRC risk, namely: changes in enterohepatic cycle; intestinal absorption decreased (due to alterations in transport); and gastrointestinal tract accumulation of lipid and toxic bile acids. Consequently, these changes cause ROS formation, DNA damage, apoptosis induction and new mutations appearance. Therefore, it is supposed that common mutant alleles from *APOA1, APOB, APOC3, CETP, NPY, PON1* and *PPARG* genes (that influence lipid metabolism and transport) can be associated with an increased SCA risk. Thus, these common polymorphisms appears be prospective biomarkers to SCA susceptibility. Most of the results described in this study have never been described and is fundamental further study for obtaining more illuminating findings. Finally, in this study it was also highlighted that primary tumour localization may be conditioned by *APOB, APOC3, CETP* and *PPARG* polymorphisms.

Chapter VI
References

6. References

1. Brenner H, Kloor M, Pox CP. Colorectal cancer. *Lancet*. 2014; 383(9927)**:**1490–1502;

2. Arvelo F, Sojo F, Cotte C. Biology of colorectal cancer. *Ecancermedicalscience*. 2015; 9(520):1–20;

3. Siegel R, Jemal A. Colorectal Cancer Facts & Figures 2014-2016. *American Cancer Society.* 2014; 1–32;

4. Karunaharamoorthy A, Becker CA. The rectum: Kenhub [Internet]. Berlim: Kenhub; 2015 [cited 2015 Oct 29]. Available from: https://www.kenhub.com/en/library/;

5. Kheirelseid EAH, Miller N, Kerin MJ. Molecular biology of colorectal cancer: Review of the literature. *American Journal of Molecular Biology*. 2013; 3(2):72–80;

6. Lipkin M, Reddy B, Newmark H, Lamprecht SA. Dietary factors in human colorectal cancer. *Annual Review of Nutrition*. 1999; 19:545–586;

7. Costa V, Casamassimi A, Ciccodicola A. Nutritional genomics era: opportunities toward a genome-tailored nutritional regimen. *Journal Nutritional Biochemistry*. 2010; 21:457–467;

8. Crous-Bou M, Rennert G, Salazar R, Rodriguez-Moranta F, Rennert HS, Lejbkowicz F, Kopelovich L, Lipkin SM, Gruber SB, Moreno V. Genetic polymorphisms in fatty acid metabolism genes and colorectal cancer. *Mutagenesis*. 2012; 27:169–176;

9. Agnoli C, Grioni S, Sieri S, Sacerdote C, Vineis P, Tumino R, Giurdanella MC, Pala V, Mattiello A, Chiodini P, Iacoviello L, Curtis AD, Cattaneo L, Duijnhoven FJB, Panico S, Krogh V. Colorectal cancer risk and dyslipidemia: A case–cohort study nested in an Italian multicentre cohort. *Cancer Epidemiology.* 2014; 38(2):144–151;

10. Rourke JSO, Johnson A, Collins P, Duignan J, Bouchier-Hayes D. An association between hypocholesterolaemia and colorectal carcinoma in an Irish population. *Gut*. 1992; 33(7):950–953;

11. Yamada K, Araki S, Tamura M, Sakai I, Takahashi Y, Kashihara H, Kono S. Relation of serum total cholesterol, serum triglycerides and fasting plasma glucose to colorectal carcinoma in situ. *International Journal of Epidemiology*. 1998; 27(5):794–798;

12. Kamano T, Mikami Y, Kurasawa T, Tsurumaru M, Matsumoto M, Kano M, Motegi K. Ratio of primary and secondary bile acids in feces: possible marker for colorectal cancer?. *Diseases of the Colon and Rectum.* 1999; 42(5)**:**668–672;

13. Cotter J. Colorectal Cancer : Portugal and the World. *Acta Médica Portuguesa*. 2013; 26(5):485–486;

14. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worlwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013 [cited 2015 Sep 9]. Available from: http://globocan.iarc.fr;

15. Bray F, Ren JS, Masuyer E, Ferlay J. Estimates of global cancer prevalence for 27 sites in the adult population in 2008. *International Journal Cancer*. 2013; 5:1133–1145;

16. Center MM, Jemal A, Ward E. International trends in colorectal cancer incidence rates. *Cancer Epidemiology, Biomarkers and Prevention.* 2009; 18(6):1688–1694;

17. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JWW, Comber H, Forman D, Bray F. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *European Journal of Cancer*. 2013; 49(6):1374–1403;

18. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA: A Cancer Journal for Clinicians.* 2015; 65(1)**:**5–29;

19. Haggar FA, Boushey RP. Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clinics in Colon and Rectal Surgery.* 2009; 22(4):191–197;

20. Stock C, Pulte D, Haug U, Brenner H. Subsite-specific colorectal cancer risk in the colorectal endoscopy era. *Gastrointestinal Endoscopy*. 2012; 75(3):621–630;

21. Center MM, Jemal A, Smith RA, Ward E. Worldwide variations in colorectal cancer. *Cancer Journal for Clinicians.* 2010; 59(6):366-378;

22. Guo P, Huang ZL, Yu P, Li K. Trends in cancer mortality in China: an update. *European Science Editing.* 2012; 38(2):35–37;

23. Davis C, Bryan J, Hodgson J, Murphy K. Definition of the mediterranean diet: A literature review. *Nutrients*. 2015; 7(11):9139–9153;

24. Norat T, Scoccianti C, Boutron-Ruault MC, Anderson A, Berrino F, Cecchini M, Espina C, Key T, Leitzmann M, Powers H, Wiseman M, Romieu I. European code against cancer 4th edition: diet and cancer. *Cancer Epidemiology*. 2015; 39(1):S56–S66;

25. Bouvard V, Loomis D, Guyton KZ, Grosse Y, Ghissassi FE, Benbrahim-Tallaa L, Guha N, Mattock H, Straif K. Carcinogenicity of consumption of red and processed meat. *Lancet Oncology*. 2015; 2045(15):1–2;

26. Gunter MJ, Leitzmann MF. Obesity and colorectal cancer: epidemiology, mechanisms and candidate genes. *Journal of Nutritional Biochemistry.* 2006; 17(3):145–56;

27. de la Chapelle A, Hampel H. Clinical Relevance of Microsatellite Instability in Colorectal Cancer. *Journal of Clinical Oncology.* 2010; 28(20):3380–3387;

28. Kwak EL, Chung DC. Hereditary colorectal cancer syndromes: an overview. *Clinical Colorectal Cancer*. 2007; 6(5):340–344;

29. Half E, Bercovich D, Rozen P. Familial adenomatous polyposis. *Orphanet Journal of Rare Diseases*. 2009; 4(1):22;

30. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: molecular basis of colorectal cancer. *New England Journal of Medicine.* 2009; 361(25):2449–2460;

31. Tejpar S, Van Cutsem E. Molecular and genetic defects in colorectal tumorigenesis. *Best Practice and Research Clinical Gastroenterology*. 2002; 16(2):171–185;

32. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell*. 1990; 61(5):759–767;

33. Navaratnam RM, Chowaniec J. Winslet MC. The molecular biology of colorectal cancer development and the associated genetic events. *Annals Royal College of Surgeons of England.* 1999; 81(5):312–319;

34. Fearon ER. Molecular genetics of colorectal cancer. *Annual Review Pathology*. 2011; 6:479–507;

35. Armaghany T, Wilson JD, Chu Q, Mills G. Genetic alterations in colorectal cancer. *Gastrointestinal Cancer Research.* 2012; 5(1):19–27;

36. Pancione M, Remo A, Colantuoni V. Genetic and epigenetic events generate multiple pathways in colorectal cancer progression. *Pathology Research International.* 2012; 2012:11;

37. Fleming M, Ravula S, Tatishchev SF, Wang HL. Colorectal carcinoma: Pathologic aspects. *Journal Gastrointestinal Oncology.* 2012; 3(3):153–173;

38. Treanor D, Quirke P. Pathology of colorectal cancer. *Clinical Oncology (Royal College of Radiologists)*. 2007; 19(10):769–776;

39. Hamilton SR, Aaltonen LA. World health organization classification of tumours. Pathology and genetics of tumours of the digestive system. *IARC Press*. 2000; 2(2):314;

40. Zhu X, Wetta H. Genetics and epigenetics in tumorigenesis: acting separately or linked?. *Journal of Clinical Medicine*. 2014; 1(4):1016;

41. Mrkonjic M, Chappell W, Pethe VV, Manno M, Daftary D, Greenwood CM, Gallinger S, Zanke BW, Knight JA, Bapat B. Association of apolipoprotein E polymorphisms and dietary factors in colorectal cancer. *British Journal of Cancer*. 2009; 100(12):1966–1974;

42. Suburu J, Gu Z, Chen H, Chen W, Zhang H, Chen YQ. Fatty acid metabolism: Implications for diet, genetic variation, and disease. *Food Bioscience.* 2013; 4:1–12;

43. Erichsen HC, Chanock SJ. SNPs in cancer research and treatment. *British Journal of Cancer.* 2004; 90(4):747–51;

44. Woutersen RA, Appel MJ, van Garderen-Hoetmer A, Wijnands MVW. Dietary fat and carcinogenesis. *Mutat. Res. Toxicol. Environ. Mutagen.* 1999; 443(1-2):111–127;

45. Huang C, Freter C. Lipid metabolism, apoptosis and cancer therapy. International *Journal of Molecular Sciences*. 2015; 16(1):924–949;

46. Rolim AEH, Henrique-Araújo R, Ferraz EG, Dultra FKAA, Fernandez LG. Lipidomics in the study of lipid metabolism: Current perspectives in the omic sciences. *Gene*. 2015; 554(2):131–139;

47. Feingold Kr, Grunfeld C. Introduction to Lipids and Lipoprotein [Internet]. *Endotext*. 2000 [cited 2015 Oct 30]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK305896/;

48. Badimón L, Vilahur G, Padró T. Lipoproteins, Platelets, and Atherothrombosis. *Revista Española de Cardiologia.* 2009; 62(10):1161–1178;

49. Corella D, Ordovas JM. Single nucleotide polymorphisms that influence lipid metabolism: Interaction with Dietary Factors. *Annual Review Nutrition.* 2005; 25:341–390;

50. Donma MM, Donma O. Apolipoproteins: biochemistry, methods and clinical significance. *Biochemical Education.* 1989; 17(2):63–68;

51. Charles MA, Kane JP. New molecular insights into CETP structure and function: a review. *Journal of Lipid Research*. 2012; 53(8):1451–1458;

52. Pihlajamäki J, Karhapää P, Vauhkonen I, Kekäläinen P, Kareinen A, Viitanen L, Pesonen U, Kallio J, Uusitupa M, Laakso M. The Leu7Pro polymorphism of the neuropeptide Y gene regulates free fatty acid metabolism. *Metabolism.* 2003; 52(5):643–646;

53. van Himbergen TM, Roest M, de Graaf J, Jansen EH, Hattori H, Kastelein JJ, Voorbij HA, Stalenhoef AF, van Tits LJ. Indications that paraoxonase-1 contributes to plasma high density lipoprotein levels in familial hypercholesterolemia. *Journal of Lipid Research.* 2005; 46(3):445–451;

54. Li T, Chiang JYL. Regulation of Bile Acid and Cholesterol Metabolism by PPARs. *PPAR Research.* 2009; 2009:501739;

55. Coban N, Onat A, Guclu-Geyik F, Komurcu-Bayrak E, Can G, Erginel-Unaltuna N. Gender-specific associations of the APOA1 -75G>A polymorphism with several metabolic syndrome components in Turkish adults. *Clinica Chimica Acta.* 2014; 431:244–9;

56. Castillo S, Tejedor D, Mozas P, Reyes G, Civeira F, Alonso R, Ros E, Pocoví M, Mata P. The apolipoprotein B R3500Q gene mutation in Spanish subjects with a clinical diagnosis of familial hypercholesterolemia. *Atherosclerosis.* 2002; 165(1):127–135;

57. Su S, Tang M, Zhang M, Cheng C, Tang X. The relationship between apolipoprotein CIII gene polymorphism and serum lipid levels in Han Chinese males. *Meta gene.* 2013; 1**:**58–64;

58. Souza DR, Nakazone MA, Pinhel MA, Alvares RM, Monaco AC, Pinheiro A, Barros CF, Cury PM, Cunrath GS, Netinho JG. Association between apolipoprotein E genotype, serum lipids, and colorectal cancer in Brazilian individuals. *Brazilian Journal of Medical and Biological Research.* 2009; 42(5):397–403;

59. Kakko S, Tamminen M, Kesäniemi YA, Savolainen MJ. R451Q mutation in the cholesteryl ester transfer protein (CETP) gene is associated with high plasma CETP activity. *Atherosclerosis*. 1998; 136(2):233–240;

60. Pac-Kożuchowska E, Krawiec P. Cholesterol ester transfer protein (CETP) gene polymorphism and selected parameters of lipid metabolism in children from families with history of cardiovascular system diseases. *Medical Science Monitor.* 2013; 19:818–825;

61. Pesonen U. NPY L7P polymorphism and metabolic diseases. *Regulatory Peptides.* 2008; 149(1-3):51–55;

62. Ahmed NS, Shafik NM, Elraheem OA, Abou-Elnoeman SE, Association of paraoxonase-1(Q192R and L55M) gene polymorphisms and activity with colorectal cancer and effect of surgical intervention. *Asian Pacific Journal of Cancer Prevention.* 2015; 16(2):803–809;

63. Slattery ML, Murtaugh MA, Sweeney C, Ma KN, Potter JD, Caan BJ, Samowitz W. PPARgamma, energy balance, and associations with colon and rectal cancer. *Nutrition and Cancer.* 2009; 51(2):155–161;

64. Moleres A, Milagro FI, Marcos A, González-Zorzano E, Campoy C, Garagorri JM, Azcona-Sanjulian MC, Martinez JA, Martin A. Common variants in genes related to lipid and energy metabolism are associated with weight loss after an intervention in overweight/obese adolescents. *Nutrición Hospitalaria.* 2014; 30(1):75–83;

65. Block R, Corsetti J, Goldenberg I, Vorobiof G, McNitt S, Ryan D, Zareba W, Moss AJ. The common apolipoprotein A-1 polymorphism -75A>G is associated with ethnic differences in recurrent coronary events after recovery from an acute myocardial infarction. *Heart International.* 2009; 4(1):e8;

66. Halley P, Kadakkuzha BM, Faghihi MA, Magistri M, Zeier Z, Khorkova O, Coito C, Hsiao J, Lawrence M, Wahlestedt C. Regulation of the apolipoprotein gene cluster by a long noncoding RNA. *Cell Reports.* 2014; 6(1):222–230;

67. Ruaño G, Seipa RL, Windemuth A, Zöllner S, Tsongalis GJ, Ordovas J, Otvos J, Bilbie C, Miles M, Zoeller R, Visich P, Gordon P, Angelopoulos TJ, Pescatello L, Moyna N, Thompson PD. Apolipoprotein A1 genotype affects the change in high density lipoprotein cholesterol subfractions with exercise training. *Atherosclerosis.* 2006; 185(1):65–69;

68. Jones MK, Catte A, Li L, Segrest JP. Dynamics of lecithin: cholesterol acyltransferase activation by apolipoprotein A-I. *Biochemistry*. 2009; 48(47):11196–11210;

69. Hamrita B, Nasr HB, Gabbouj S, Bouaouina N, Chouchane L, Chahed K. Apolipoprotein A1 -75 G/A and +83 C/T polymorphisms: susceptibility and prognostic implications in breast cancer. *Molecular Biology Reports.* 2011; 38(3):1637–1643;

70. Tachibana M, Ohkura Y, Kobayashi Y, Sakamoto H, Tanaka Y, Watanabe J, Amikura K, Nishimura Y, Akaqi K. Expression of apolipoprotein A1 in colonic adenocarcinoma. *Anticancer Research.* 2003; 23(5b):4161–4167;

71. Smith JD, Brinton EA, Breslow JL. Polymorphism in the human apolipoprotein A-I gene promoter region. Association of the minor allele with decreased production rate in vivo and promoter activity in vitro. *Journal of Clinical Investigation.* 1992; 89(6):1796–1800;

72. Hsu MC, Lee KT, Hsiao WC, Wu CH, Sun HY, Lin IL, Young KC. The dyslipidemia-associated SNP on the APOA1/C3/A5 gene cluster predicts post-surgery poor outcome in Taiwanese breast cancer patients: a 10-year follow-up study. *BMC Cancer.* 2013; 13:330;

73. Dixit M, Choudhuri G, Saxena R, Mittal B. Association of apolipoprotein A1-C3 gene cluster polymorphisms with gallstone disease. *Canadian Journal Gastroenterology and Hepatology.* 2007; 21(9):569–75;

74. Juo SH, Wyszynski DF, Beaty TH, Huang HY, Bailey-Wilson JE. Mild association between the A/G polymorphism in the promoter of the apolipoprotein A-I gene and apolipoprotein A-I levels: a meta-analysis. *American Journal of Medical Genetics.* 1999; 82(3):235–241;

75. Yang MH, Rampal S, Sung J, Choi Y-H, Son HJ, Lee JH, Kim YH, Chang DK, Rhee P-L, Kim JJ, Rhee JC, Chun H-K, Guallar E, Cho J. The association of serum lipids with colorectal adenomas. *American Journal of Gastroenterology.* 2013; 108(5):833–841;

76. Zhang X, Zhao XW, Liu DB, Han CZ, Du LL, Jing JX, Wang Y. Lipid levels in serum and cancerous tissues of colorectal cancer patients. *World Journal of Gastroenterolgy.* 2014; 20(26):8646–8652;

77. Manchekar M, Richardson PE, Forte TM, Datta G, Segrest JP, Dashti N. Apolipoprotein B-containing lipoprotein particle assembly: lipid capacity of the nascent lipoprotein particle. *Journal of Biological Chemistry.* 2004; 279(38):39757–39766;

78. Haas ME, Attie AD, Biddinger SB. The regulation of ApoB metabolism by insulin. *Trends in Endocrinology and Metabolism.* 2013; 24(8):391–397;

79. Soufi M, Sattler AM, Maerz W, Starke A, Herzum M, Maisch B, Schaefer JR. A new but frequent mutation of apoB-100-apoB His3543Tyr. *Atherosclerosis.* 2004; 174(1):11–16;

80. Doo M, Won S, Kim Y. Association between the APOB rs1469513 polymorphism and obesity is modified by dietary fat intake in Koreans. *Nutrition.* 2014; 31(5):653-658;

81. Vrablík M, Ceska R, Horínek A. Major Apolipoprotein B-100 mutations in lipoprotein metabolism and atherosclerosis. *Physiol. Res.* 2001; 50(4):337–343;

82. Kalina A, Császár A, Czeizel AE, Romics L, Szabóki F, Szalai C, Reiber I, Németh A, Stephenson S, Williams RR. Frequency of the R3500Q mutation of the apolipoprotein B-100 gene in a sample screened clinically for familial hypercholesterolemia in Hungary. *Atherosclerosis.* 2001; 154(1):247–251;

83. Shen H, Damcott CM, Rampersaud E, Pollin TI, Horenstein RB, McArdle PF, Peyser PA, Bielak LF, Post WS, Chang YP, Ryan KA, Miller M, Rumberger JA, Sheedy PF, Shelton J, O'Connell JR, Shuldiner AR, Mitchell BD. Familial Defective Apolipoprotein B-100 and Increased Low- Density Lipoprotein Cholesterol and Coronary Artery Calcification in the Old Order Amish Haiqing. *Archives of Internal Medicine.* 2013; 170(20):1850–1855;

84. Tai DY, Pan JP, Lee-Chen GJ. Identification and haplotype analysis of apolipoprotein B-100 Arg3500--> Trp mutation in hyperlipidemic Chinese. *Clinical Chemistry.* 1998; 44(8):1659–1665;

85. Eroğlu Z, Selvi N, Kosova B, Biray C, Kumral E, Topçuoğlu N, Kayikçioğlu M. Absence of apolipoprotein B-3500 mutation in Turkish patients with coronary and cerebrovascular atherosclerosis. *Anadolu Kardiyoloji Dergisi.* 2008; 8(1):7–9;

86. Jacobsen N, Fenger M, Bentzen J, Rasmussen SL, Jakobsen MH, Fenstholt J, Skouv J. Genotyping of the apolipoprotein B R3500Q mutation using immobilized locked nucleic acid capture probes. *Clinical Chemistry.* 2002; 48(4):657–660;

87. Clemente-Postigo M, Queipo-Ortuño M, Valdivielso P, Tinahones FJ, Cardona F. Effect of apolipoprotein C3 and apolipoprotein A1 polymorphisms on postprandial response to a fat overload in metabolic syndrome patients. *Clinical Biochemistry.* 2010; 43(16-17):1300–1304;

88. Jong MC, Hofker MH, Havekes LM. Role of ApoCs in lipoprotein metabolism: functional differences between ApoC1, ApoC2, and ApoC3. *Arteriosclerosis Thrombosis and Vascular Biology.* 1999; 19(3):472–484;

89. Song Y, Zhu L, Richa M, Li P, Yang Y, Li S. Associations of the APOC3 rs5128 polymorphism with plasma APOC3 and lipid levels: a meta-analysis. *Lipids Health Dis.* 2015; 14(32):1–14;

90. Mahley RW, Rall SC. Apolipoprotein E: far more than a lipid. *Annual Review of Genomics and Human Genetics.* 2000; 1:507–537;

91. Saadat M. Apolipoprotein E (APOE) polymorphisms and susceptibility to breast cancer: a meta-analysis. *Cancer Research and Treatment.* 2012; 44(2):121–126;

92. Mazzotti DR, Singulane CC, Ota VK, Rodrigues TP, Furuya TK, de Souza FJ, Cordeiro BG, de Oliveira Amaral CM, Chen ES, Jacomini A, de Arruda Cardoso Smith M, Borsatto-Galera B. Association of APOE, GCPII and MMP9 polymorphisms with common diseases and lipid levels in an older adult/elderly cohort. *Gene.* 2014; 535(2):370–375;

93. Watson MA, Gay L, Stebbings WS, Speakman CT, Bingham SA, Loktionov A. Apolipoprotein E gene polymorphism and colorectal cancer: gender-specific modulation of risk and prognosis. *Clinical Science.* 2003; 104(5):537–545;

94. Sehayek E, Shefer S, Nguyen LB, Ono JG, Merkel M, Breslow JL. Apolipoprotein E regulates dietary cholesterol absorption and biliary cholesterol excretion: studies in C57BL/6 apolipoprotein E knockout mice. *Proceedings of the National Academy of Sciences of the U. S. A.* 2000; 97(7):3433–3437;

95. Cibeira GH, Giacomazzi J, Aguiar E, Schneider S, Ettrich B, DE Souza CI, Camey S, Caleffi M, Weber B, Ashton-Prolla P, Moriguchi EH. Apolipoprotein E genetic polymorphism, serum lipoprotein levels and breast cancer risk: a case-control study. *Molecular and Clinical Oncology.* 2014; 2(6):1009–1015;

96. Ifere GO, Desmond R, Demark-Wahnefried W, Nagy TR. Apolipoprotein E gene polymorphism influences aggressive behavior in prostate cancer cells by deregulating cholesterol homeostasis. *International Journal Oncology.* 2013; 43(4):1002–1010;

97. De Feo E, Simone B, Persiani R, Cananzi F, Biondi A, Arzani D, Amore R, D'Ugo D, Ricciardi G, Boccia S. A case-control study on the effect of Apolipoprotein E genotypes on gastric cancer risk and progression. *BMC Cancer.* 2012; 12:494;

98. Al-Meghaiseeb ES, Al-Otaibi MM, Al-Robayan A, Al-Amro R, Al-Malki AS, Arfin M, Al-Asmari AK. Genetic association of apolipoprotein E polymorphisms with inflammatory bowel disease. *World Journal Gastroenterology.* 2015; 21(3)**:**897–904;

99. Hill SA, Nazir DJ, Jayaratne P, Bamford KS, McQueen MJ. Mutations in cholesteryl ester transfer protein and hepatic lipase in a North American population. *Clinical Biochemistry.* 1997; 30(5):413–418;

100. Tsai MY, Li N, Sharrett AR, Shea S, Jacobs DR Jr, Tracy R, Arnett D, Arends V, Post W. Associations of Genetic Variants in ATP-Binding Cassette A1 and Cholesteryl Ester Transfer Protein and Differences in Lipoprotein Subclasses in the Multi-Ethnic Study of Atherosclerosis. *Clinical Chemistry.* 2009; 55(3):481–488;

101. Tsai MY, Johnson C, Kao WH, Sharrett AR, Arends VL, Kronmal R, Jenny NS, Jacobs DR Jr, Arnett D, O'Leary D, Post W. Cholesterol ester transfer protein genetic polymorphisms, HDL cholesterol, and subclinical cardiovascular disease in the Multi-Ethnic Study of Atherosclerosis. *Atherosclerosis*. 2013; 200(2):359–367;

102. Ma J, Nordman S, Möllsten A, Falhammar H, Brismar K, Dahlquist G, Efendic S, Gu HF. Distribution of neuropeptide Y Leu7Pro polymorphism in patients with type 1 diabetes and diabetic nephropathy among Swedish and American populations. *European Journal. Endocrinology.* 2007; 157(5):641–645;

103. Beck B. Neuropeptide Y in normal eating and in genetic and dietary-induced obesity. *Philosophical Transactions of the Royal Society of London B Biological Sciences.* 2006; 361(1471):1159–1185;

104. Bhaskar LV, Thangaraj K, Pardhasaradhi G, Kumar KP, Singh L, Rao VR. Neuropeptide Y gene polymorphisms are not associated with obesity in a South Indian population. *European Journal of Clinical Nutrition.* 2010; 64(8):868–872;

105. Kakko T, Jaakkola U, Raitakari OT, Kallio J. Inflammatory effects of blood leukocytes: association with vascular function in neuropeptide Y proline 7-genotyped type 2 diabetes patients. *Diabetes and Vascular Disease Research.* 2011; 8(3):221–228;

106. Masoudi-Kazemabad A, Jamialahmadi K, Moohebati M, Mojarrad M, Dehghan-Manshadi R, Forghanifard MM, Akhlaghi S, Ferns GA, Ghayour-Mobarhan M. High frequency of Neuropeptide Y Leu7Pro polymorphism in an Iranian population and its association with coronary artery disease. *Gene.* 2012; 496(1):22–27;

107. Jaakkola U, Kakko T, Seppälä H, Vainio-Jylhä E, Vahlberg T, Raitakari OT, Kallio J. The Leu7Pro polymorphism of the signal peptide of neuropeptide Y (NPY) gene is associated with increased levels of inflammatory markers preceding vascular complications in patients with type 2 diabetes. *Microvascular Research.* 2010; 80:433–439;

108. Ilveskoski E, Viiri LE, Mikkelsson J, Pörsti I, Lehtimäki T, Karhunen PJ. Neuropeptide Y signal peptide Pro7 substitution protects against coronary artery atherosclerosis: the Helsinki Sudden Death Study. *Atherosclerosis.* 2008; 199(2):445–450;

109. Salminen M, Lehtimäki T, Fan YM, Vahlberg T, Kivelä, SL. Leucine 7 to proline 7 polymorphism in the neuropeptide Y gene and changes in serum lipids during a family-based counselling intervention among school-aged children with a family history of CVD. *Public Health Nutrition.* 2008; 11(11):1156–1162;

110. Masoudi-Kazemabad A, Jamialahmadi K, Moohebati M, Mojarrad M, Manshadi RD, Akhlaghi S, Ferns GA, Ghayour-Mobarhan M. Neuropeptide Y Leu7Pro polymorphism associated with the metabolic syndrome and its features in patients with coronary artery disease. *Angiology.* 2013; 64(1):40–45;

111. Ruscica M, Dozio E, Boghossian S, Bovo G, Martos Riaño V, Motta M, Magni P. Activation of the Y1 receptor by neuropeptide Y regulates the growth of prostate cancer cells. *Endocrinology.* 2006; 147(3):1466–1473;

112. Medeiros PJ, Al-Khazraji BK, Novielli NM, Postovit LM, Chambers AF, Jackson DN. Neuropeptide Y stimulates proliferation and migration in the 4T1 breast cancer cell line. *International Journal of Cancer.* 2012; 131(2):276–286;

113. Goswami B, Tayal D, Gupta N, Mallika V. Paraoxonase: a multifaceted biomolecule. *Clinica Chimica Acta.* 2009; 410(1-2):1–12;

114. Mackness M, Mackness B. Human paraoxonase-1 (PON1): gene structure and expression, promiscuous activities and multiple physiological roles. *Gene.* 2015; 567(1)**:**12–21;

115. Ceron JJ, Tecles F, Tvarijonaviciute A. Serum paraoxonase 1 (PON1) measurement: an update. *BMC Veterinary Research.* 2014; 10:74;

116. Kresanov P, Vasankari T, Ahotupa M, Kaikkonen J, Hutri-Kähönen N, Juonala M, Kähönen M, Lehtimäki T, Viikari J, Raitakari OT. Paraoxonase-1 and oxidized lipoprotein lipids. The Cardiovascular Risk in Young Finns Study. *Atherosclerosis.* 2015; 241(2):502–506;

117. ou-Bonafonte J, Gabás-Rivera C, Navarro M, Osada J. PON1 and Mediterranean diet. *Nutrients.* 2015; 7(6):4068–4092;

118. Balci H, Genc H, Papila C, Can G, Papila B, Yanardag H, Uzun H. Serum lipid hydroperoxide levels and paraoxonase activity in patients with lung, breast, and colorectal cancer. *Journal Clinical Laboratory Analysis.* 2012; 26(3):155–160;

119. Gutierrez A, Ratliff EP, Andres AM, Huang X, McKeehan WL, Davis RA. Bile acids decrease hepatic paraoxonase 1 expression and plasma high-density lipoprotein levels via FXR-mediated signaling of FGFR4. *Arteriosclerosis Thrombosis and Vascular Biology.* 2005; 26(2):301–306;

120. Zhang M, Xiong H, Fang L, Lu W, Wu X, Huang ZS, Wang YQ, Cai ZM, Wu S. Paraoxonase 1 (PON1) Q192R gene polymorphism and cancer risk: a meta-analysis based on 30 publications. *Asian Pacific Journal of Cancer Prevention.* 2015; 16(10):4457–4463;

121. Jiang J, Gajalakshmi V, Wang J, Kuriki K, Suzuki S, Nakamura S, Akasaka S, Ishikawa H, Tokudome S. Influence of the C161T but not Pro12Ala polymorphism in the peroxisome proliferator-activated receptor-gamma on colorectal cancer in an Indian population. *Cancer Science.* 2005; 96(8):507–512;

122. Slattery ML, Murtaugh MA, Sweeney C, Ma KN, Potter JD, Caan BJ, Samowitz W. PPARgamma, energy balance, and associations with colon and rectal cancer. *Nutrition and Cancer.* 2009; 51(2):155–161;

123. Youssef J, Badr M. Peroxisome proliferator-activated receptors and cancer:

challenges and opportunities. *British Journal Pharmacology.* 2011; 164(1):68–82;

124. Murtaugh MA, Ma KN, Caan BJ, Sweeney C, Wolff R, Samowitz WS, Potter JD, Slattery ML. Interactions of peroxisome proliferator–activated receptor {gamma} and diet in etiology of colorectal cancer. *Cancer Epidemiol Biomarkers and Prevention.* 2005; 14(5):1224–1230;

125. Park SS, Park SK, Lim JH, Choi YH, Kim WJ, Moon SK. Esculetin inhibits cell proliferation through the Ras/ERK1/2 pathway in human colon cancer cells. *Oncology Reports.* 2011; 25(1):223–230;

126. Wei Z, Han G, Bai X. Effect of proliferator-activated receptor-γ Pro12Ala polymorphism on colorectal cancer risk: a meta-analysis. *Medical Science Monitor.* 2015; 21:1611–1616;

127. Hagland HR, Søreide K. Cellular metabolism in colorectal carcinogenesis: Influence of lifestyle, gut microbiome and metabolic pathways. *Cancer Letters.* 2015; 356(2): 273–80;

128. Bernstein C, Payne CM, Bernstein H. Bile acids: promoters or carcinogens in colon cancer? *Journal of Carcinogenesis and Mutagenesis.* 2011; 2:101e;

129. Debruyne PR, Bruyneel EA, Li X, Zimber A, Gespach C, Mareel MM. The role of bile acids in carcinogenesis. *Mutation Research/Fundamental and Molecular Mechanisms Mutagenesis.* 2001; 480-481:359–369;

130. Nagengast F, Grubben MJ, van Munster IP. Role of bile acids in colorectal carcinogenesis. *European Journal Cancer.* 1995; 31(7-8):1067–1070;

131. Degirolamo C, Modica S, Palasciano G, Moschetta A. Bile acids and colon cancer: Solving the puzzle with nuclear receptors. *Trends in Molecular Medicine.* 2011; 17:564–572;

132. Green MR, Sambrook J. Molecular cloning - A laboratory Manual . *Cold Spring Harbour Laboratory*. 2012; Vol 1. New York. USA;

133. Slattery ML. Single nucleotide polymorphisms in sporadic colorectal cancer. *Current Colorectal Cancer Reports.* 2006; 2(4):200–205.

134. Schwab US, Agren JJ, Valve R, Hallikainen MA, Sarkkinen ES, Jauhiainen M, Karvonen MK, Pesonen U, Koulu M, Uusitupa MI, Savolainen MJ. The impact of the leucine 7 to proline 7 polymorphism of the neuropeptide Y gene on postprandial lipemia and on the response of serum total and lipoprotein lipids to a reduced fat diet. *European Journal of Clinical Nutrition.* 2002; 56(2):149–156;

135. Gu SJ, Guo ZR, Zhou ZY, Hu XS, Wu M. PPAR α and PPAR γ polymorphisms as risk factors for dyslipidemia in a Chinese Han population. *Lipids in Health and Disease.* 2014; 13:23;

136. Staels B, Fonseca VA. Bile acids and metabolic regulation: mechanisms and clinical responses to bile acid sequestration. *Diabetes Care.* 2009; 32(2):S237–S245;

137. Li T, Chiang JYL. Bile acid signaling in liver metabolim and diseases. *Journal of Lipids.* 2012; 2012;

138. Jacobs RJ, Voorneveld PW, Kodach LL, Hardwick JC. Cholesterol metabolism and colorectal cancers. *Current Opinion in Pharmacology.* 2012; 12(6):690–695;

139. Yao X, Tian Z. Dyslipidemia and colorectal cancer risk: a meta-analysis of prospective studies. *Cancer Causes Control.* 2015; 26(2):257–268;

140. Gong Z, Xie D, Deng Z, Bostick RM, Muga SJ, Hurley TG, Hebert JR. The PPAR{gamma} Pro12Ala polymorphism and risk for incident sporadic colorectal adenomas. *Carcinogenesis.* 2005; 26(3):579–585;

141. Baptissart M, Vega A, Maqdasy S, Caira F, Baron S, Lobaccaro JM, Volle DH. Bile acids: from digestion to cancers. *Biochimie.* 2013; 95(3):504–517;

142. Martinez-Augustin O, Sanchez de Medina F. Intestinal bile acid physiology and pathophysiology. *World Journal of Gastroenterology.* 2008; 14(37):5630–5640;

143. Vlahcevic ZR, Heuman DM, Hylemon PB. Regulation of Bile Acid Synthesis. *Hepatology.* 1991; 13(3):590–600;

144. Bernstein C, Holubec H, Bhattacharyya AK, Nguyen H, Payne CM, Zaitlin B, Bernstein H. Carcinogenicity of deoxycholate, a secondary bile acid. *Archives of Toxicology.* 2011; 85(8):863–871;

145. Ryan K, Phillips A, Vousden K. Regulation and function of the p53 tumor suppressor protein. *Current Opinion in Cell Biology.* 2001; 13(3):332–337;

146. Ajouz H, Mukherji D, Shamseddine A. Secondary bile acids: an underrecognized cause of colon cancer. *World Journal of Surgical Oncology.* 2014; 12:164;

147. Sniderman AD, Zhang Z, Genest J, Cianflone K. Effects on apoB-100 secretion and bile acid synthesis by redirecting cholesterol efflux from HepG2 cells. *Journal of Lipid Research.* 2003; 44(3):527–532;

148. Lin Y, Havinga R, Schippers IJ, Verkade HJ, Vonk RJ, Kuipers F. Characterization of the inhibitory effects of bile acids on very-low-density lipoprotein secretion by rat hepatocytes in primary culture. *Biochemical Journal*. 1996; 316(2):531–538;

149. Lowenfels AB. Is increased cholesterol excretion the link between low serum cholesterol and colon cancer? Nutrition and Cancer. 1983; 4(4):280–284;

150. Mackness B, Beltran-Debon R, Aragones G, Joven J, Camps J, Mackness M. Human tissue distribution of paraoxonases 1 and 2 mRNA. *IUBMB Life*. 2010; 62(6):480–482;

151. Janani C, Ranjitha Kumari BD. PPAR gamma gene--a review. *Diabetes Metabolic Syndrome*. 2015; 9(1):46–50;

152. Frattini M, Balestra D, Suardi S, Oggionni M, Alberici P, Radice P, Costa A, Daidone MG, Leo E, Pilotti S, Bertario L, Pierotti MA. Different genetic features associated with colon and rectal carcinogenesis. *Clinical Cancer Research*. 2004; 10(12):4015–4021;

153. Lancet D, Safran M, Rosen N, Stelzer G, Iny-Stein T, Bahir I, Rapparport N, Twik M, Fishilevich S, Kohn A, Edri O, Plaschkes I, Lieder I, Kaplan S, Mazor Y. Genecard [Internet]. Israel: Weizmann Institute of Science; 1996-2015 [cited 2015 Oct 30]. Available from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=APOC3&keywords=APOC3;

154. Werner, A., Kuipers, F. & Verkade, H. J. Fat Absorption and Lipid Metabolism in Cholestasis. Madame Curie Biosci. Database [Internet]. Austin Landes Biosci. 1–17. 2000 [cited 2015 Oct 30]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK6420/.

Websites

http://www.genecards.org/cgi-bin/carddisp.pl?gene=APOA1&keywords=APOA1

http://www.genecards.org/cgi-bin/carddisp.pl?gene=APOB&keywords=APOB

http://www.genecards.org/cgi-bin/carddisp.pl?gene=APOC3&keywords=APOC3

http://www.genecards.org/cgi-bin/carddisp.pl?gene=APOE&keywords=APOE

http://www.genecards.org/cgi-bin/carddisp.pl?gene=CETP&keywords=CETP

http://www.genecards.org/cgi-bin/carddisp.pl?gene=NPY&keywords=NPY

http://www.genecards.org/cgi-bin/carddisp.pl?gene=PON&keywords=PON

http://www.genecards.org/cgi-bin/carddisp.pl?gene=PPARG&keywords=PPARG

Chapter VII
Appendix

7. Appendix

Table 20. Hardy-Weinberg Equilibrium to common lipid metabolism genes polymorphisms in control population.

Hardy-Weinberg Equilibrium		
SNP	**Controls**	
	χ^2	***P-value***
***APOA1* -75G>A**	0,99	0,26
***APOB* R3500Q**	1	0,96
***APOC3* C3175G**	0,97	0,2
***APOC3* T3206G**	0,86	0,02
***APOE* Cys112Arg**	1	0,84
***APOE* Arg158Cys**	1	0,78
***CETP* G279A**	0,91	0,33
***CETP* R451Q**	0,97	0,6
***NPY* Leu7Pro**	1	0,84
***PON1* Q192R**	0,98	0,03
***PPARG* Pro12Ala**	1	0,45